STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE SHEEP

I. THE OVULATION RATE OF THE EWE AS AFFECTED BY THE PLANE OF NUTRITION [1]

RICHARD T. CLARK
University of Minnesota

INTRODUCTION

The phenomenon of twinning in sheep has occasioned considerable interest on the part of both the sheep breeders and those interested in the physiology of reproduction. As a result of their observations over a long period of time, the practice of increasing the amount and variety of nutrients fed to the ewes some time before breeding has become part of the routine of an efficiently managed flock. The term 'flushing' has been used to describe this practice and it is regarded as a necessary procedure in order to insure a high degree of fertility.

OBJECT

The object of this investigation was to attempt to determine the fundamental factors influencing the phenomenon of twinning and more specifically to focus attention on the effects of increased nutrients on the rate of ovulation. A review of the

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The author is indebted to Prof. W. H. Peters, chief of the Division of Animal Husbandry, for providing the necessary experimental animals.

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literature reveals no experiments of a similar nature where accurate records of the ovulation rate were made.

HISTORICAL

It has long been recognized that the reproductive system of the sheep can be influenced very materially by changes in environment. Aristotle observed the increased fertility of sheep when they were subjected to a favorable environment.

Darwin stated that domestic animals tend to breed at more frequent periods and to produce a larger number of offspring than wild animals of the same species.

Heape (1899) reported on the question of the fertility of various breeds of sheep, taking into consideration the effects of environment. He concluded, 1) that environment plays an important part in the fertility of a breed, 2) that the fertility of a flock depends to quite an extent on its management, 3) that certain seasons seem to be more conducive to high fertility than others, and, 4) the plane of nutrition influences the ability of the sheep to breed.

White and Roberts ('27) reported that the lambing average of the Welsh Mountain breed is much higher when the ewes are kept in lowland districts rather than in mountain districts.

Marshall ('08) investigated the results of flushing. The data indicated that the lambing percentage, as a general rule, was highest in the ewes which had been flushed. The twin births appeared in the early part of the lambing season, indicating that during the first part of the breeding season the ewes were apparently in a condition conducive to high fertility. He concluded that there must be a general increase in the ovarian metabolism as a result of the stimulation afforded by the special food supply. Marshall and Potts ('24) reported an increased lamb crop as a result of flushing and demonstrated that breeds of sheep exhibit marked differences in the frequency of multiple births.

Nichols (''26) presented data supporting the findings of Marshall and also brought out the differences between breeds with respect to reproductive ability. Similar differences were reported by Richter ('26). Marshall ('22) showed that the normal percentage of ova discharged at any single oestrous period is not appreciably in excess of the usual percentage of births at the lambing season and suggested that a scarcity of twin births at that time is the direct result of a low number of ripe follicles in the ovary at the time of mating. Hammond ('21), writing on the factors controlling fertility in domestic

animals, divided these factors into two groups: 1) "the factors which control the number of eggs shed and, 2) the factors which control the number of eggs which develop to reach birth size." He demonstrated that the percentage of atrophic foetuses is high in pigs and tame rabbits where the number of eggs shed is high and that it is low in wild rabbits and sheep where the number of eggs shed is low. Hammond found that most of the atrophic foetuses which he observed in sheep had atrophied at an early stage in development and concluded that atrophy is the result of forces present in the foetus itself. From limited observations made on some early stages in the embryonic development of the pig and more extensive investigations concerning the embryology of the sheep, I am inclined to agree with Hammond's generalization.

Marshall ('08), Roberts ('21), Bell ('23), Nichols ('24), White and Roberts ('27), Marshall and Potts ('24) and Johansson ('32) have reported that a higher percentage of twin pregnancies is obtained when ewes are mated early in the fall than when mating is delayed until later in the breeding season. Jones and Rouse ('20), Roberts ('21), Marshall and Potts ('24), Wriedt ('25), Clark ('31), and Johansson ('32 a) have shown that the maximum lambing average was reached when the ewes were 5 or 6 years of age. The age of the sire was found by Marshall and Potts ('24) to have no influence on the frequency of multiple births.

Marshall ('08), writing on the inheritance of fertility in sheep, stated "that fertility is a character which can be inherited is a fact which admits of no doubt and many flockmasters testify to the truth of this statement." Newman ('17) stated that both monozygotic and dizygotic twinning are characters capable of being inherited as unit characters and that they could be made racial or specific characters by selective breeding. Bell ('23), after conducting a series of experiments, was uncertain whether the twin bearing tendency in sheep was really a hereditary character. Castle ('24), reporting from the records of the Bell flock, was inclined to believe that the effect of heredity on twinning was of secondary importance to that of environment.

Heape (1899) and Nichols ('26) have presented adequate data to demonstrate very clearly that certain breeds of sheep possess the ability to reproduce consistently at a higher rate than other breeds; consequently, we have good reason for believing that such differences in reproductive ability are due to fundamental genetic differences between the breeds. It is

true that environmental influences do play a part, as in the expression of all truly genetic factors, in the manifestation of these breed differences, but environmental differences alone will not explain the great variation in reproductive ability shown by the various breeds. The studies which have been made regarding the mode of twin production are largely of a statistical nature. In the second paper of this series, embryological evidence will be submitted to substantiate in part the theories which have been advanced on the basis of statistical data on the sex ratio of twin births in sheep. The only embryological evidence for monozygotic twinning in sheep was presented by Assheton (1898). He wrote, "In the sheep (ovis) I found some years ago a blastocyst at the stage just before the formation of the embryonal areas with two distinct ectodermic masses lying within the trophoblast." Regarding this observation, Newman ('17) concluded that very likely double embryos of this type do not develop very far. If monozygotic twinning does occur to any extent in sheep, then we have a right to expect a ratio of twins on the basis of sex to show a marked departure from a 1 MM : 2 MF : 1 FF ratio. Such a deviation from this ratio is known to be true of human twins.

That this expectation is not realized in sheep and that we find no departure from the theoretical 1 : 2 : 1 ratio has been demonstrated by the ratios which have been presented in the literature. Clark ('31) reported a ratio of 129 MM : 273 MF : 121 FF from the flock records of the University of Minnesota and concluded that the occurrence of monozygotic twins must be extremely rare, if they do occur at all. Chapman and Lush ('32) presented a ratio of 87 MM : 184 MF : 90 FF and drew a similar conclusion. Johansson ('32 a) presented much more extensive data, his observed frequencies of sex combinations in 5088 sets of twins being as follows: 1164 MM : 2685 MF : 1239 FF. His ratio shows an excess of opposite sexed twins, this excess being statistically significant.

EXPERIMENTAL PROCEDURE AND DATA

Experiment 1. In this experiment, which was conducted during the breeding season 1931–32, forty grade western ewes were used as experimental subjects. Thirty-eight of them were 2½ years old, the other two being 3½ years old. They were very uniform in type, showing a fairly high degree of Merino breeding. They were allotted into two equal groups on the basis of initial weight, condition, and morphological

characteristics. After being allotted, group I was subjected to a relatively high plane of nutrition, whereas group II was fed a ration designed merely to maintain them as close as possible to their initial weight.

Group I at the end of the first week was consuming per day 50 pounds of alfalfa hay and 20 pounds of a grain mixture consisting of 500 parts oats, 75 parts oil cake, and 50 parts bran. In addition to this ration they were allowed access to a rye pasture for 2 or 3 hours each day.

Group II was fed 20 pounds of alfalfa hay and 40 pounds of prairie hay.

Both groups were placed on the experimental rations on October 28th, and the first matings were made on November 16th. In the mating of the ewes the system of hand breeding was followed. In this way it was possible to record the exact time that mating occurred. The recording of the matings and the details of flock management were carried out by the writer in person. Each ewe was mated twice, the time of the first service being recorded. The time of destruction was determined by the stage in embryonic development which was desired. Prior to slaughtering, the weight of the ewe was recorded in order to obtain a measure of her performance under the given experimental conditions and to relate that to the number of ova liberated. Immediately after slaughtering the reproductive organs were examined and the number of corpora lutea recorded.

TABLE 1

Summary showing relation of rate of gain to ovulation

	EWES SHOWING DOUBLE OVULATIONS	EWES SHOWING SINGLE OVULATIONS	TOTAL GAIN IN WEIGHT, POUNDS	TOTAL EWE DAYS ON FEED	AVERAGE RATE OF GAIN PER DAY, POUNDS
Experiment 1 (1931–32)					
Flushed	8	12	245.4	1074	0.228
Unflushed		20	27.7	1262	0.022
Experiment 2 (1932–33)					
Flushed	10	10	136.8	646	0.212
Unflushed	12	8	31.7	828	0.038

TABLE 2

Summary of ovulation records (corpora lutea)

	RIGHT OVARY	LEFT OVARY	TOTAL	AVERAGE
Experiment 1 (1931–32)				
Group I (flushed)	15	13	28	1.4
Group II	12	8	20	1.0
	27	21		
Experiment 2 (1932–33)				
Group I (flushed)	12	18	30	1.5
Group II	19	15	34	1.7
	31	33		

It will be observed from the data furnished that a reasonable degree of success was secured in causing group I to gain in weight and to keep group II on a purely maintenance basis. Table 1 shows that the daily rate of gain of the flushed group was 0.228 pound per day per ewe whereas the unflushed group was maintained very close to the original weight showing an average rate of gain of only 0.022 pound per day. In table 2 it will be observed that the ovulation average for the flushed group was 1.4, while that of the unflushed group was 1.0.

In view of these results it would seem that there is a definite relationship between increase in body weight and the number of ova liberated. This would substantiate the findings of Marshall and Potts ('24) who even went so far as to state "that in order to produce the largest number of twins, ewes should gain at least 7 pounds a head during the breeding season." The flushed group in this experiment showed an average gain of slightly more than 12 pounds.

It is a recognized fact that the Merino type of sheep does not exhibit the twinning tendency to as great a degree as most of the mutton breeds. This factor must be recognized in the interpretation of the results secured. The ovulation record of the flushed group begins to approach the upper limit of the lambing average for this breed.

No difference was found between the groups with respect to time of coming in heat. The double ovulations were fairly well distributed throughout the entire season.

Experiment 2. In this experiment forty pure bred Shropshire ewes from the University of Minnesota flock were used, again allotting twenty ewes in each group. Having accurate pedigree records of these ewes as well as their own lambing performance, it was possible to balance the groups with respect to, 1) initial weight, 2) lambing average, 3) ewes that were triplets, twins, or singles, 4) whether the dams of these ewes were triplets, twins, or singles, and, 5) age of the ewes.

Group I was gradually brought up to a daily consumption of 50 pounds of alfalfa hay and 20 pounds of grain mixture of the same proportions as were used in the previous season, while group II was fed 60 pounds of alfalfa hay per day.

The groups were started on feed on the fourteenth of October, and the first matings were made on the twenty-sixth of October. Table 1 reveals that in this experiment the daily rate of gain of the flushed group was 0.212 pound per day per ewe, while the ovulation average as shown by table 2 was 1.5. The unflushed group was maintained relatively close to the initial weight, the average rate of gain being 0.038 pound per day while the ovulation average was 1.7.

These data reveal no advantage in favor of the group that gained in weight; in fact, there is a slight advantage in favor of the group that was fed a maintenance ration. In spite of the limited numbers within the groups, it can be stated that the data reveal no positive relationship between gain in weight and ovulation record.

The ovulation averages for both groups fall within the limits of the lambing averages for the Shropshire breed which have been published. No difference was observed between the groups with respect to time of coming in heat. The double ovulations occurred more or less uniformly throughout the breeding season.

GENERAL DISCUSSION

In order to reconcile what might appear to be contradictory results and conclusions when experiment 1 and experiment 2 are discussed collectively, it is necessary to bear in mind the factor of the condition of the ewes at the beginning of the experimental period.

The forty western ewes in experiment 1 were in a relatively thin, though a thrifty condition, and therefore in an ideal state to demonstrate the effects of a rising plane of nutrition. As a result, the flushed group ovulated at a higher rate than the unflushed group. On the other hand, in experiment 2 the forty Shropshire ewes were in a relatively high condition at the beginning of the experiment. The flushed group did not ovulate at as high a rate as the unflushed, even though they did show appreciable gains in body weight. It can be inferred that the condition of the ewes at the beginning of the experiment is an important factor in determining the response to the imposed environmental conditions, and the indirect effect of this response, the ovulation record. From these results it would seem that the broad generalization that a rising condition, indicated by an increase in body weight, results in an increased ovulation rate, must be tempered by giving due emphasis to the condition of the ewes prior to the flushing period. For this reason it would be futile to apply this practice to a flock in high condition; such a case might arise where a long period intervened between weaning and breeding, especially if the ewes were on a high plane of nutrition.

One of the advantages ascribed to flushing is that the ewes come in heat earlier, resulting in a more uniform lamb crop. Such a conclusion cannot be drawn from the data secured in both experiments 1 and 2. On the contrary, the ewes in both the flushed and unflushed groups came in heat more or less uniformly throughout the breeding season. It is true that different results might have been secured if the flushing period had come prior to the establishment of rhythmic oestrous cycles, tending to bring a greater proportion of the ewes to

the ram at approximately the same time. Marshall and Potts ('24) observed no advantage in flushing from the standpoint of bringing the ewes into heat earlier, so that the data in experiments 1 and 2 support their findings.

From table 2 it will be observed that the right and left ovaries are equally potential. It will also be observed from table 2 that the ovulation rates compare very closely with the lambing averages for the breeds concerned, indicating that Hammond's contention that the percentage of ova unaccounted for is relatively low in sheep is correct. The data reveal that in both experiments the double ovulations occurred throughout the entire breeding season.

CONCLUSIONS

1. The practice of flushing will lead to a higher ovulation rate, provided the ewes are not in high condition to start with.
2. Gain in weight of itself is not necessarily indicative of a higher ovulation rate.
3. Flushing appears to have no material effect in bringing ewes into 'heat' faster, provided their oestrous cycles have already been established.
4. The cases of double ovulation occurred fairly uniformly throughout the entire breeding season.
5. The ovulation rates are approximately the same as the lambing averages for the breeds that were used in the experiments.

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STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE SHEEP

II. THE CLEAVAGE STAGES OF THE OVUM [1]

RICHARD T. CLARK
University of Minnesota

ONE TEXT FIGURE, ONE GRAPH AND FOUR PLATES (TWENTY FIGURES)

INTRODUCTION

Comparatively few studies of mammalian embryos have been conducted for the purpose of collecting specimens of rather definitely known ages. This is particularly true of our larger farm animals for the reasons that considerable labor and expense is necessary to handle those species.

In this paper the stages up to and slightly beyond the period of implantation are described. Embryological evidence is presented regarding the relatively rare occurrence of monozygotic twinning in sheep. This evidence substantiates the statistical data referred to in the first paper of this series, which indicated that twins in sheep were of dizygotic origin.

HISTORICAL

The literature relative to the embryological development of the sheep is very limited. The earlier writings are presented largely as a matter of historical interest.

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The photomicrographs were made by Mr. V. P. Hollis, director of the Photographic Laboratory of the University of Minnesota.

Von Baer (1828) cited the investigations of Haller and his student, Kuhlmann. They examined ewes at regular intervals after mating, but much to their astonishment found nothing prior to the twelfth day. From that time on they observed increasing amounts of mucus. On the seventeenth day they found the first traces of the embryo, and on the nineteenth day an embryo of considerable size.

Von Baer (1828) reported the recovery of an ovum from the fallopian tube. He noted that the nucleus was very loose and shrunken and the yolk particles or droplets were held together on the outer surface. He wrote of the zona pellucida as a 'skin' which is quite thick and encircles the yolk.

Hartman et al. ('31) stated that Hausmann and Bischoff had observed tubal ova of the sheep about the middle of the last century. I have not been able to get Bischoff's original manuscript but have examined Hausmann's and find no description of tubal ova. Hausmann (1840) did, however, conduct a rather extensive investigation on the physiology of reproduction in the sheep. He reported that the height of heat lasts only 24 hours, sometimes only 16, and that the duration of the oestrous cycle is close to 3 weeks. He believed that the graafian follicle did not burst in the event of the ewe not being mated, and postulated the necessity of coitus for the liberation of the ovum. In certain cases, however, he believed that a ewe which had passed through several heat periods, without being bred, might eventually ovulate without coition and lead to the formation of a false corpus luteum. Hausmann destroyed ewes at $1\frac{1}{4}$ hours, 5 hours and 50 minutes, 6 hours, 12 hours, and 20 hours after mating. On examining the ovaries he found that at $1\frac{1}{4}$ hours they contained ripe follicles but none had burst. He found the same condition in the ovaries of the ewe that had gone 5 hours and 50 minutes and attempted to recover an ovum from one of the ripe follicles but was unsuccessful. He repeated the experiment with a ewe that had gone 6 hours after mating without ovulating and was successful in securing what he calls an 'ovulum graafianum.' Presumably this was an ovum because he uses 'graafische Bläschen' to describe the graafian follicle. In the other two cases he was unsuccessful; in fact, in the 20-hour case he found a corpus luteum already beginning to form. In none of those cases does he refer to searching for tubal ova. He does indicate that he attempted to study sperm travel by slitting the uterus and making smears from portions of the horns.

Bonnet (1884) reported his inability to secure the stages in the development of the sheep prior to the twelfth day after mating.

In 1898, Assheton presented his classical work entitled, "The segmentation of the ovum of the sheep, with observations on the hypothesis of a hypoblastic origin for the trophoblast." Before reviewing this paper it would seem fitting, in view of the unsuccessful efforts of the earlier investigators, to quote a short paragraph contained in another paper by Assheton (1898 a):

"There were signs of the discharge of two separate ova from one of the ovaries. I only found this one specimen, which was obtained from the horn of the uterus in connection with the ovary showing the two corpora lutea. Anyone who has attempted to find the early embryos of the sheep will know how difficult a task it is and will not be surprised that I found but one specimen out of the two expected on this occasion."

I must admit that I am in perfect agreement with his opinion. That Assheton must have possessed a great deal of patience is certain because he obtained ova from the fallopian tube by scraping off the whole of the mucous membrane with a scalpel, placing the scrapings on a slide and examining this material under a microscope. How laborious his task must have been can only be appreciated by one who has spent hour after hour searching through dishes of saline solution washed from the fallopian tubes. To obtain embryos from the uterus he resorted to a technique which has been followed by later workers in this field, with slight modifications, and which involved filling the uterus with fluid and allowing this fluid to flow from the cervical end into suitable receptacles, controlling this flow with a ligature or clamp. Assheton, unfortunately, was unable to study this embryonic material in the living condition, as he used 0.25 or 0.5 per cent chromic acid for a washing fluid. He destroyed forty-one sheep and obtained forty specimens. He did not obtain any specimens between the fertilized, unsegmented ovum and a stage showing six cells. He was unable to identify a polar body. He wrote that "the ovum of the sheep is large, measuring as much as 0.15 mm. in diameter or including the zona radiata 0.18 mm. while fresh." In discussing the passage of the ovum through the oviduct, he stated that it did not acquire a covering of albumin during its sojourn in the oviduct. He presented a graded series of embryos from the second to the eleventh day of development. Regarding time intervals, he writes, "The dates which I

give are only approximate. I am not sure that there is so much variation in the time that may elapse between the moment of sexual union and fertilization as in the case of the pig. It is, however, very difficult to obtain any one given stage. Circumstances did not permit of a continuous watch being kept upon the flock of sheep. The dates which I give are not from my own observations, but are derived from the information given me by the shepherd."

Assheton stated that the ova usually reach the uterus early on the third day, when they are in the eight-cell stage. In sections of an eight-cell stage he distinguished a cell which was lighter in appearance than the other seven cells, although the eight blastomeres were not very different in size. Even though he was unable to find this condition in sections of other eight-cell stages, he postulated that perhaps as early as this the future epiblast and hypoblast are being differentiated.

Allen et al. ('31) reported the recovery of ova in one-cell, four-cell, and four- to six-cell stages. Ovulation was not observed before $2\frac{3}{4}$ hours after the first signs of oestrus. Between that time and 50 hours unsegmented ova were recovered. Four-cell stages were recovered at $53\frac{1}{2}$ hours; four- to six-cell stages at 66 to 98 hours. Regarding the size of the ova the following data were presented: outside diameter, 0.125 to 0.167 mm.; thickness of zona pellucida, 0.009 to 0.012 mm., and diameter of vitellus, 0.091 to 0.131 mm.

PROCEDURE AND DATA

The animals used in this investigation were those included in experiments 1 and 2 of the first paper in this series.

The flock was watched very carefully during the entire period of the investigation and readings taken at the time the ewes showed the first signs of oestrus. In this species it is almost impossible to use any sign of vascularization and swelling of the external genitalia as an index of whether an animal is in heat. Indeed, the extent to which the external genitalia became vascularized varies greatly in different individuals. The only satisfactory criterion is that of introducing a ram into the flock and observing the willingness of the ewe to associate with this male. Suitable precautions were taken to prevent this ram from mating any ewes that might

be in heat by wrapping a closely woven sack around his belly. By visiting the flock at 4-hour intervals during the day, it was possible to tell reasonably accurately at what time of day a particular ewe came in heat and when her period of desire terminated.

In order to insure a high percentage of successful matings and to have an accurately timed series, the approximate time of ovulation was ascertained by destroying a number of ewes at different intervals during the period of 'heat.' It was found to occur rather late in the 'heat' period. Using this as a base, matings were made at the close of the second day after the ewe first showed signs of oestrus. The question of an optimum time of mating has been pointed out by Marshall and Hammond ('32), in the case of the mare, and it would seem that the success of the experiment reported in this paper was due in no small part to the fact that matings were made at the time when the ova would be proceeding down the fallopian tubes.

Immediately after the ewe was slaughtered and the dressing of the carcass completed the ovaries were examined and the number of corpora lutea recorded. The reproductive tract was then removed to the laboratory and dissected for study. In securing tubal ova the technique reported by Corner ('21) was followed. It consists essentially of directing a stream of saline solution through the fallopian tube. The tube is dissected from its mesosalpinx and straightened, for the straighter the tube the greater the likelihood of success. In detaching the tube it is well to include a small portion of the cornu as this allows the operator to direct the pipette used in flushing to better advantage. After experimenting with different sizes of pipettes one tapering to a bore of 1 mm. with a small medicine dropper bulb was found to be the most desirable size. The flushing was always done toward the infundibulum, and with few exceptions the ovum was flushed out in the first 5 cc. of saline solution. It is highly desirable to use a small amount of fluid and to prevent any of the fluid running down the outside of the tube because it will carry

with it a certain amount of cellular debris which will make the task of searching for the ovum more difficult. The washings were caught in a small watch glass with a very concave surface. Such a receptacle aids in the search for the ovum, as it is possible to rotate the small amount of fluid by manipulating the watch glass under the microscope; and since the ovum lies on the bottom of the watch glass, it will tend to roll to the center. The highly refractive appearance of the zona pellucida makes the identification of the ovum fairly simple once the investigator knows what to look for. In washing uteri a larger pipette tapering to a bore of 4 mm. with a bulk of 20 cc. capacity was found to be the most suitable size. The fluid was introduced at the tubal end of the cornu and the overflow caught in a large watch glass at the cervical end.

Profiting by the experience of Heuser, Streeter and Gregory all photomicrographs of the specimens were made in Locke's solution while they were still alive and fresh. (A human hair mounted on a fine hemostatic clamp makes a very desirable instrument for the manipulation of the ova.)

The first two or three ova that were obtained were photographed in the small watch glass into which they had been washed from the fallopian tube. After acquiring the ability to manipulate the ova from dish to dish, using a fine pipette, it was found advisable to place the ovum which was to be photographed in a micro culture slide. The cell was then completely filled with saline solution and a cover glass placed on top. In this way movement of the saline was almost negligible and clearer pictures of the specimens resulted. The most desirable and representative pictures are presented in this paper, those which were considered indistinct being discarded. Immediately after photographing, the specimens were fixed in Bouin's fluid. A few of the ova were stained in Delafield's hematoxylin and prepared as whole mounts. The technique outlined by Gregory ('30) was followed.

Measurement of ova. Table 1 shows that the ovum, as measured by the diameter of the zonal cavity, does not change

appreciably during the period in which its zona pellucida remains intact.

TABLE 1

Measurement of ova

EWE NO.	OVUM NO.	STAGE OF DEVELOPMENT	AGE OF OVUM (POST-COITUM)	DIAMETER OF ZONAL CAVITY	THICKNESS OF ZONA PELLUCIDA
			hours	*mm.*	*mm.*
512	1	Unsegmented	$4\frac{1}{2}$	0.146	0.014
255	2	Unsegmented	$17\frac{1}{2}$	0.153	0.016
24–26	5	Unsegmented	$32\frac{1}{4}$	0.149	0.011
19–26	17	Unsegmented	$37\frac{1}{3}$	0.156	0.014
	18	Unsegmented		0.153	0.015
22–30	6	Unsegmented	$38\frac{3}{4}$	0.144	0.014
648119	26	2-cell	$39\frac{1}{3}$	0.153	0.013
	27	2-cell		0.151	0.013
663058	19	4-cell	$42\frac{1}{4}$	0.142	0.013
	20	8-cell		0.142	0.013
272	3	8-cell	44	0.160	0.015
	4			0.148	0.012
8–31	10	16-cell	$65\frac{1}{3}$	0.146	0.014
607956	7	16-cell +	77	0.151	0.014
	8	16-cell +		0.149	0.015
450	12	16- to 32-cell	96	0.141	0.012
	13	16- to 32-cell		0.137	0.013
218	14	32-cell approximate	$113\frac{1}{2}$	0.135	0.014
	15	32-cell approximate		0.135	0.013
8–29	16	32 cells +	$138\frac{3}{4}$	0.152	0.015
			Average	0.147	0.014

It was observed that a pair of ova from a single ewe appeared to be more similar in their measurements than were pairs of ova from different ewes.

To test this variability between ewes an analysis of the variance was made. The data are presented in table 2.

TABLE 2

Analysis of the variance between pairs of ova

VARIATION DUE TO SIZE OF OVA OF	DEGREES OF FREEDOM	SUM OF SQUARES	VARIANCE	z
Different ewes	6	742.7084	123.7847	
Same ewe	7	88.5000	12.6429	1.1408
Total	13	831.2084		

Since the observed value of Z (1.1408) exceeds the value of Z for the 1 per cent point (0.9864), for $N_1 = 6$ and $N_2 = 7$ degrees of freedom, it may be concluded that the chances are in excess of 99:1 that the observed differences in size of ova between the ewes are real.

GENERAL DISCUSSION

To measure mammalian ova accurately one can make use of photomicrographs of known magnification, where the living ova are immersed in an appropriate medium. By following this method in this investigation, it was found that the ovum of the sheep, as measured by the diameter of the zonal cavity, ranged from 0.135 to 0.160 mm. with an average diameter of 0.147 mm. The zona pellucida ranged in thickness from 0.011 to 0.016 mm. with an average thickness of 0.014 mm.

Hartman ('29) stated that most of the dimensions which had been given previously for mammalian ova were too low. His estimate of the sheep ovum is 0.120 mm., considerably lower than the average diameter found in this experiment. It will be recalled that Assheton (1898) estimated the sheep ovum at 0.150 mm. or 0.180 mm. if the zona radiata is included. His figure of 0.150 mm. is remarkably close to the average diameter, 0.147 mm., of the ova measured in this study. From a comparative standpoint, it is of interest to note the measurements of ova of several other forms which have appeared in the literature. They are presented in table 3.

TABLE 3

Comparative measurements of ova

FORM	INVESTIGATOR	DIAMETER OF ZONAL CAVITY	THICKNESS OF ZONAL
		mm.	*mm.*
Guinea pig	Squier ('32)	0.095 (0.087–0.107)	0.012 (0.009–0.0149)
Rabbit	Gregory ('30)	0.128 (0.110–0.146)	0.019 (0.011–0.023)
Pig	Heuser & Streeter (29)	0.130	0.015
Cow	Hartman et al. ('31)	0.135–0.140	0.012–0.015
Sheep	(Own data)	0.147 (0.135–0.160)	0.014 (0.011–0.016)
Sheep	Allen et al. ('31)	0.125–0.167 (outside diameters)	0.009–0.012

The ovum of the sheep is larger than that of any of the forms presented in this brief table. There is, however, a remarkable degree of similarity among the ova presented above which becomes all the more impressive when we consider the relative body sizes of the various species.

No cumulus cells were attached to any of the ova which were examined, the only adherent material which had to be 'brushed' off prior to photography being small particles of dust or detritus which settled in the saline.

The sheep ovum in the unfertilized or unsegmented condition resembles other mammalian ova in its morphology. A study of table 1 shows, however, that there is very little variation between the different ova as regards the thickness of the zona pellucida. This cannot be said of the ova which Gregory ('30) measured in his study of the rabbit. Squier's ('32) data on guinea pig ova show uniformity in thickness of zona pellucida.

The sheep ovum presents the characteristic appearance of mammalian ova consisting of droplets of lipoid-like material distributed throughout in a uniform manner. The ovum proper, even in the earliest specimen (fig. 2), which was recovered from a ewe destroyed 4½ hours post-coitum, does not quite fill the zonal cavity. This perivitelline space shows a certain amount of granular material.

No evidence of polar bodies was found in the ova which were examined in this investigation. It will be recalled that Assheton (1898) was also unable to find them. Allen et al. ('31) identified polar bodies in several sheep ova. The ovum presented in figure 1 represents an attempt to study the reactions of spermatozoa toward a fertilized, unsegmented ovum which was immersed in a saline solution. The ovum was observed for 2 hours after the diluted semen had been introduced. No spermatozoa, in the vicinity of the ovum, were observed to deviate from their paths; but those which were obstructed in their travel by the ovum struck and adhered to the zona pellucida, and a few sperm heads were enveloped by the zona.

The rate of travel of the ovum down the fallopian tube was not investigated. Anderson ('27) has demonstrated in the pig that the ova pass through the ampulla rapidly and the isthmus very slowly. Parker ('31) is of the opinion that the ova pass down the fallopian tubes as a result of peristalsis and ciliary action.

In figure 3 there is presented an unsegmented ovum which, after staining and mounting, had cleared sufficiently to show the union of the two pronuclei. About this time in the investi-

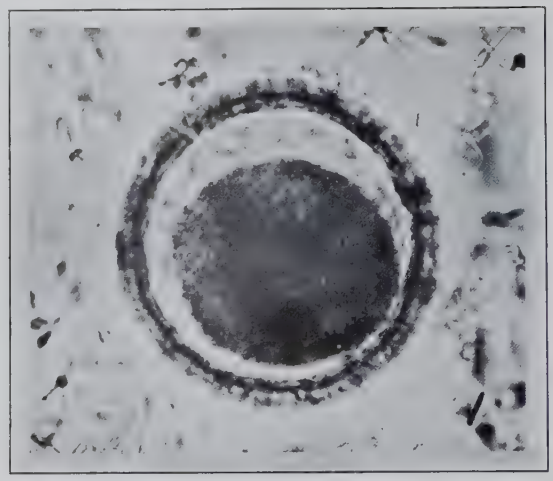


Fig. 1 Unsegmented ovum, 17½ hours post-coitum, with spermatozoa introduced artificially. × 260.

gation an attempt was being made to recover two-cell stages. Eventually, by destroying a ewe 39⅓ hours post-coitum, a pair of two-cell stages were obtained (fig. 5). As far as is known they constitute the first initial cleavage stages of the sheep which have ever been presented.[2] They have a common characteristic in that the two blastomeres in each case are quite unequal. Van Beneden originally thought that the inequality in size of these blastomeres indicated the different roles they would play in ontogenetic development. At present

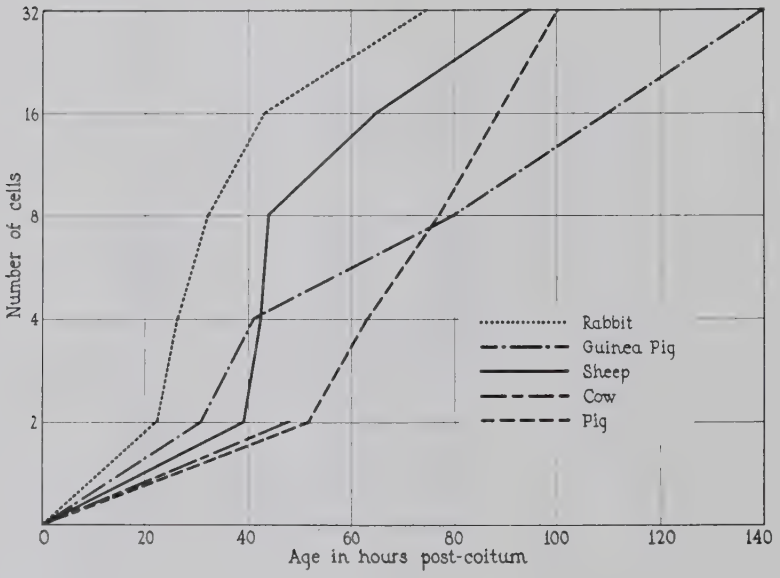
[2] Since this paper was written Mackenzie et al. (Proc. Am. Soc. An. Prod., 1933, pp. 278–281) have reported the recovery of a two-cell stage.

it is thought that this question of size is merely a matter of chance separation of cytoplasmic material in the progress of the initial division. A study of the four-cell stage in figure 6 reveals an indication of size difference between the different pairs of cells, but it is too slight to be of any great significance. Regarding this four-cell stage, it will be observed that cleavage has evidently occurred at right angles judging by the crossed arrangement of the blastomeres. Gregory ('30) demonstrated a similar condition in the second cleavage of the rabbit ovum. Both of the ova in figure 6 were obtained from the same fallopian tube. The corresponding ovary showed two corpora lutea of different size. From then on a close watch was kept in the cases of double ovulations where the two corpora lutea appeared to be of different sizes, particularly if they were located on opposite ovaries. In the cases observed, which were relatively few it is true, the larger corpus luteum indicated a more advanced stage in segmentation. A study of graph 1 will show that the first three divisions follow each other very rapidly, so that one is not justified in criticizing Assheton for his inability to secure any specimens between the unsegmented ovum and the six-cell stage.

It will be observed in graph 1 that of the five forms considered, the rabbit is more precocious in its cleavage rate than any of the others. Outside of the first two divisions, the sheep follows the rabbit at an interval of from 12 to 22 hours. While the guinea pig reaches the four-cell stage a little ahead of the sheep, it is much slower in reaching the blastocyst stage; in fact, considerably slower than either the sheep or the pig.

Before passing on to the more advanced stages, it should be mentioned that during the investigation some difficulty was encountered in getting a few of the ewes bred because their period of desire was of such short duration that they refused to take the ram at the time scheduled after first being observed in heat. Two such cases are discussed in this paper. They were held by hand and the ram was allowed to mate them

forcibly. The two-cell stages presented in figure 5 were obtained from ewe no. 648119 as the result of such a mating. The same is true of the blastocyst stage obtained from ewe no. 26–69 and shown in figure 20. These cases are of particular interest in view of the theory advanced by Parker ('31) that in the rabbit the sperms are deposited in the vagina of the female then transferred by muscular action to the uterus through its cervix. He is of the opinion that this action



Graph 1

constitutes a part of the female orgasm, and yet in this experiment we have two cases where the female certainly did not exhibit any outward symptoms of orgasm; in fact in both cases it took a considerable amount of physical effort on the part of the operator to hold the ewe a sufficient length of time to allow service by the ram. Parker ('31) stated that under certain conditions the muscular contraction may be replaced by the locomotor properties of the sperms themselves. This would be the only plausible explanation of the two cases that are reported above.

Assheton (1898) stated that the ova entered the uterus early in the third day, when they are in the eight-cell stage. Heuser and Streeter ('29) reported that in the case of the pig the ova enter the uterus in the four-cell stage sometime between the close of the second day and the beginning of the third day after mating. Three sixteen-cell stages were recovered from the fallopian tubes in this experiment, one at 65 hours and the two presented in figures 10 and 11 at 77 hours. The ova, therefore, enter the uterus some time during the fourth day for the stages presented in figure 12 were obtained from the uterus at 96 hours.

A study of figures 10 and 11 reveals differences in cell size which are quite marked, sufficiently so to be interpreted as the first concrete evidence of the early differentiation of the trophoblast cells. In the pig Heuser and Streeter ('29) found that differentiation of the trophoblast cells begins as early as the fifth day after insemination at which time the ovum is in about the sixteen-cell stage. It seems, therefore, that the trophoblast is more precocious in the sheep. Assheton's theory regarding the hypoblastic origin of the trophoblast will not be considered in this investigation, but from figures 10 and 11 it would appear that his interpretation is a reasonable one.

It is difficult to determine just how many cells are contained in the specimens presented in figures 12 and 13. They show no apparent variation in size as measured by the diameter of the zonal cavity.

Figures 14 and 15 show two trophoblast stages where the vesicles are somewhat flattened and wrinkled. They were extremely fragile; the vesicles themselves resembled a very thin veil. The stage presented in figure 16 is the only case where there is an apparent variation in the sequence of development presented in this investigation. Theoretically, it should precede the two trophoblast stages in figures 14 and 15. This variation in timing can hardly be explained on the basis of variation in time of fertilization alone. It is a very interesting case in that it represents a stage in development

when the ovum is about to be divested of its outer covering. The segmentation cavity appears as a slightly clearer area.

Figure 17 represents a stage somewhat further advanced than the two specimens represented in figures 14 and 15. The vesicle is distended into the shape of a sphere having filled with fluid in the meantime. This specimen always maintained its spherical form. The inner cell mass is very evident, being represented as a small disc of cells on the periphery of the vesicle. The cells of the vesicle itself are rather large, distinct cells, somewhat flattened in appearance. From this stage on considerable difficulty was experienced in securing representative stages. That it is a difficult gap to bridge is shown by the dearth of implantation stages recorded in the literature. However, a 10-day 19-hour specimen was secured and it is presented in figure 18. This blastocyst had very definitely attached itself to the uterine mucosa and was located in one of the folds on the dorsal surface of the uterus about 7 cm. from the cervical end. The vesicle was very transparent and as a consequence, difficult to locate. It was freed by manipulating the surrounding regions of the endometrium with dissecting needles. The character of the vesicle was quite different from that represented in figure 17. The individual cells can no longer be distinguished. That this vesicle is a chorion which is about to elongate was indicated by the presence of an inner sac which became apparent after fixation. It separated from the outer trophodermal layer, particularly in the region close to the blastodisc. Development from then on is rapid as is shown in the specimens presented in figures 19, 20 and 21. The chorion elongates very rapidly in its attempt to establish itself.

Hammond ('14), Corner ('21 a) and Warwick ('26) have shown that in the case of the pig migration of ova from one uterine horn to the other takes place. In the course of this investigation no evidence of such a phenomenon was found, the ova always being located in the corresponding horn.

Marshall ('22) stated that the oestrous cycle in the sheep ranges from 13 to 21 days. Darlow and Hawkins ('31) observed 168 cycles and found that they ranged from 13 to 21 days, with an average length of 17.3 days. The range in this investigation was from 13 to 18 days, with an average of 16.4. The length of the period of desire or 'heat' ranged from less than 24 hours to 72 hours with the majority falling between 36 and 48 hours.

CONCLUSIONS

1. The ovum of the sheep as measured by the diameter of the zonal cavity is 0.147 mm. and the average thickness of the zona pellucida is 0.014 mm.

2. The data presented in table 2 show that pairs of ova liberated by individual ewes are more similar in their measurements than the ova from other ewes of the same breed.

3. The ovum is entirely free of cumulus cells when it commences its journey down the fallopian tube.

4. The pronuclei are in the process of union 32 hours post-coitum.

5. The initial cell division takes place between 38 and 39 hours post-coitum, the blastomeres being unequal in size.

6. The two-cell stages presented in this study are, as far as is known, the first that have been recovered from the sheep.

7. The second and third cell divisions follow the initial one very quickly, the four- and eight-cell stages being found at 42 hours post-coitum. The cell divisions forming the four-cell stage have taken place at right angles as shown by the crossed arrangement of the blastomeres.

8. No polar bodies were observed in any of the ova.

9. Sixteen-cell stages were recovered from the fallopian tube at 65 hours post-coitum and the ova pass into the uterus between 77 and 96 hours.

10. Differentiation of the trophoblast cells is observed in the sixteen-cell stage.

11. The thirty-two-cell stage is reached about 96 hours post-coitum.

12. Up to the close of the sixth day the ovum does not change in size.

13. The ovum loses its zona pellucida some time between the close of the sixth day and the close of the eighth.

14. By the close of the ninth day the trophoblast has changed to a spherical vesicle distended with fluid.

15. Implantation takes place between the close of the ninth day and the close of the eleventh.

16. No instance of monozygotic twinning was found.

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PLATE 1

EXPLANATION OF FIGURES

2 Unfertilized sheep ovum, 4½ hours post-coitum. × 223.

3 Unsegmented ovum, 32¼ hours post-coitum, showing fusion of pronuclei. Bouin; Delafield's hematoxylin. × 369.

4 Unsegmented ova, 37⅓ hours post-coitum, obtained from same fallopian tube. × 223.

5 Two-cell stages, 39⅓ hours post-coitum, obtained from opposite fallopian tubes. Note unequal blastomeres. × 223.

6 Four- and eight-cell stages, 42¼ hours post-coitum, obtained from same fallopian tube. Note crossed arrangement of blastomeres. × 223.

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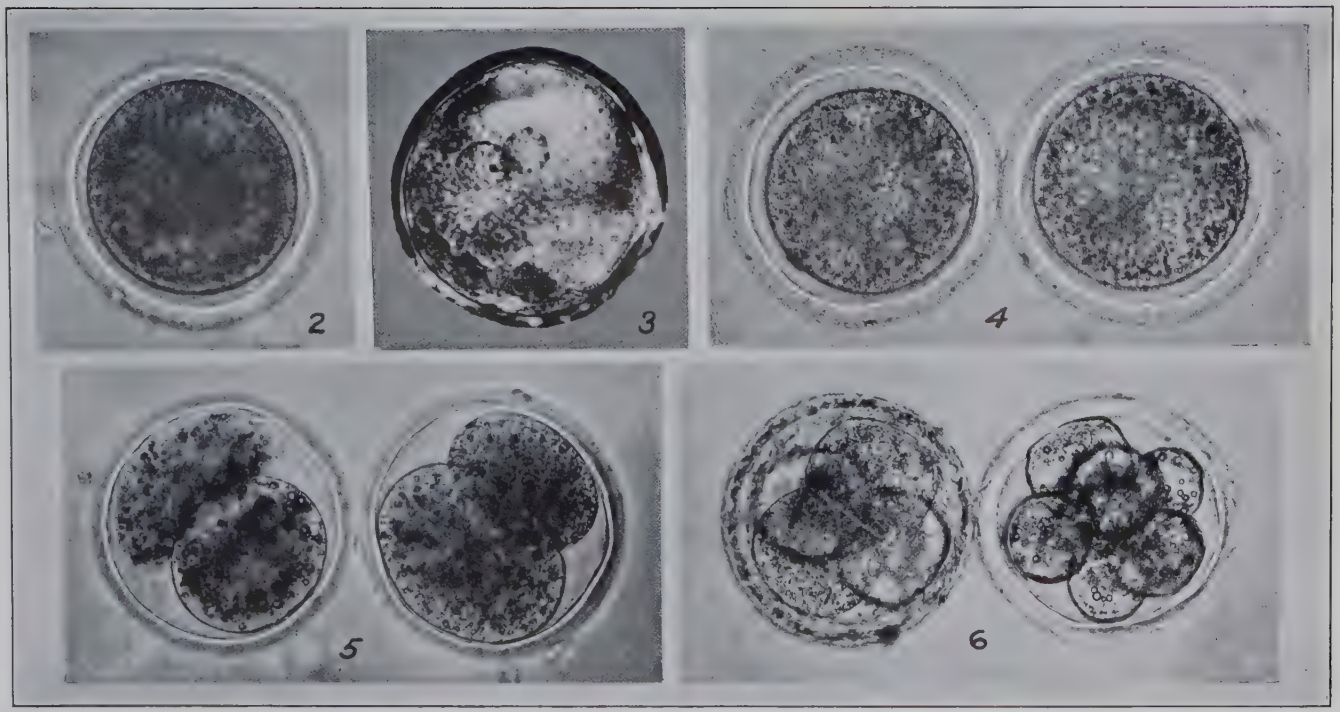

PLATE 2

EXPLANATION OF FIGURES

7 Six-cell stage, 44 hours post-coitum, obtained from same fallopian tube as the eight-cell stage shown in figure 8. × 244.

8 Eight-cell stage, 44 hours post-coitum. × 244.

9 A whole mount of the eight-cell stage shown in figure 8. Bouin; Delafield's hematoxylin. × 467.

10 Sixteen-cell stage, 77 hours post-coitum, showing differentiation of trophoblast. Obtained from the same ewe, but the opposite fallopian tube, as the stage shown in figure 11. × 244.

11 Sixteen cells +, 77 hours post-coitum, showing differentiation of trophoblast. × 244.

12 Sixteen- to thirty-two-cell stages, 96 hours post-coitum. First ova washed from uterus. × 244.

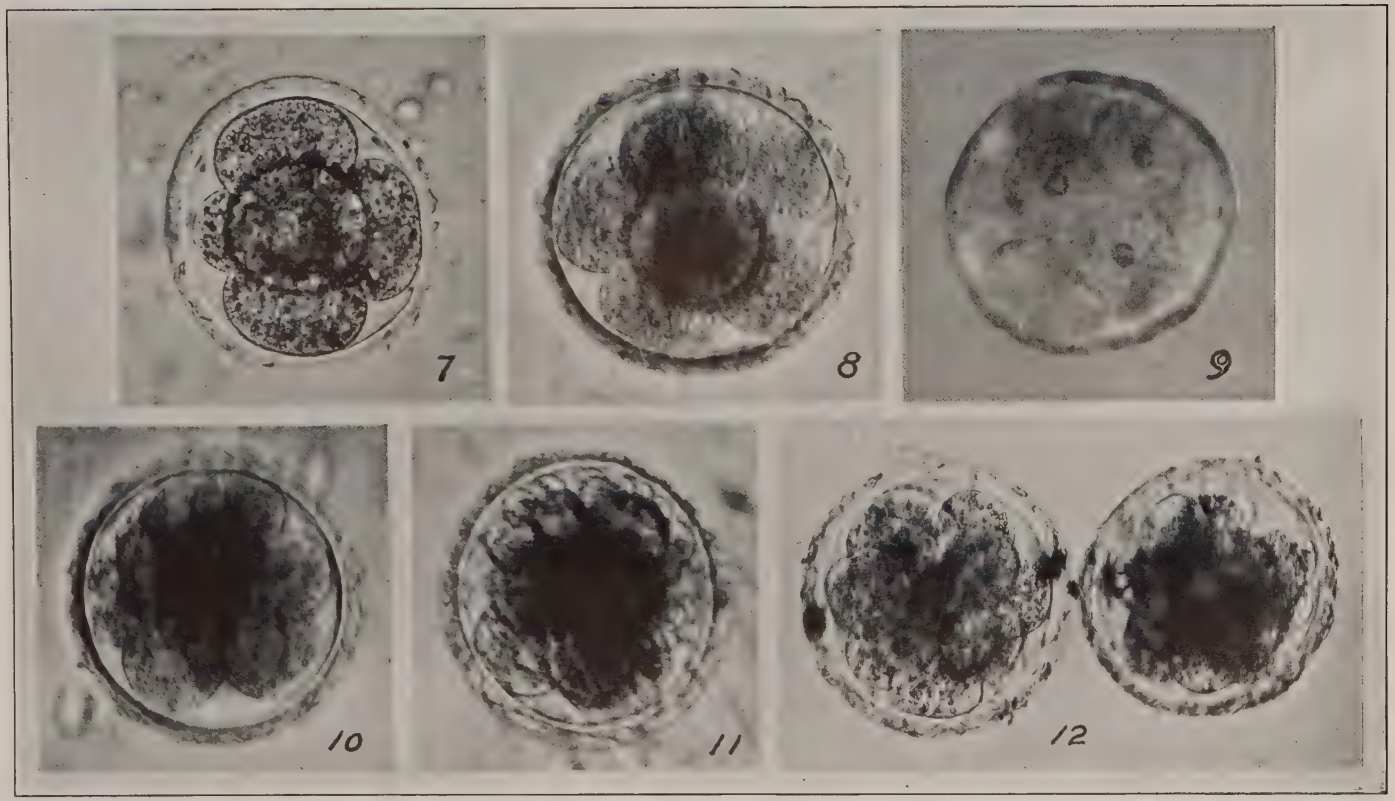
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PLATE 3

EXPLANATION OF FIGURES

13 Thirty-two-cell stages approximately, 4 days 17½ hours post-coitum. × 260.

14 and 15 Trophoblast stages, 6 days 21½ hours post-coitum. × 260.

16 Ovum about to lose its zona pellucida, 8 days 1¾ hours post-coitum. Note appearance of segmentation cavity. × 260.

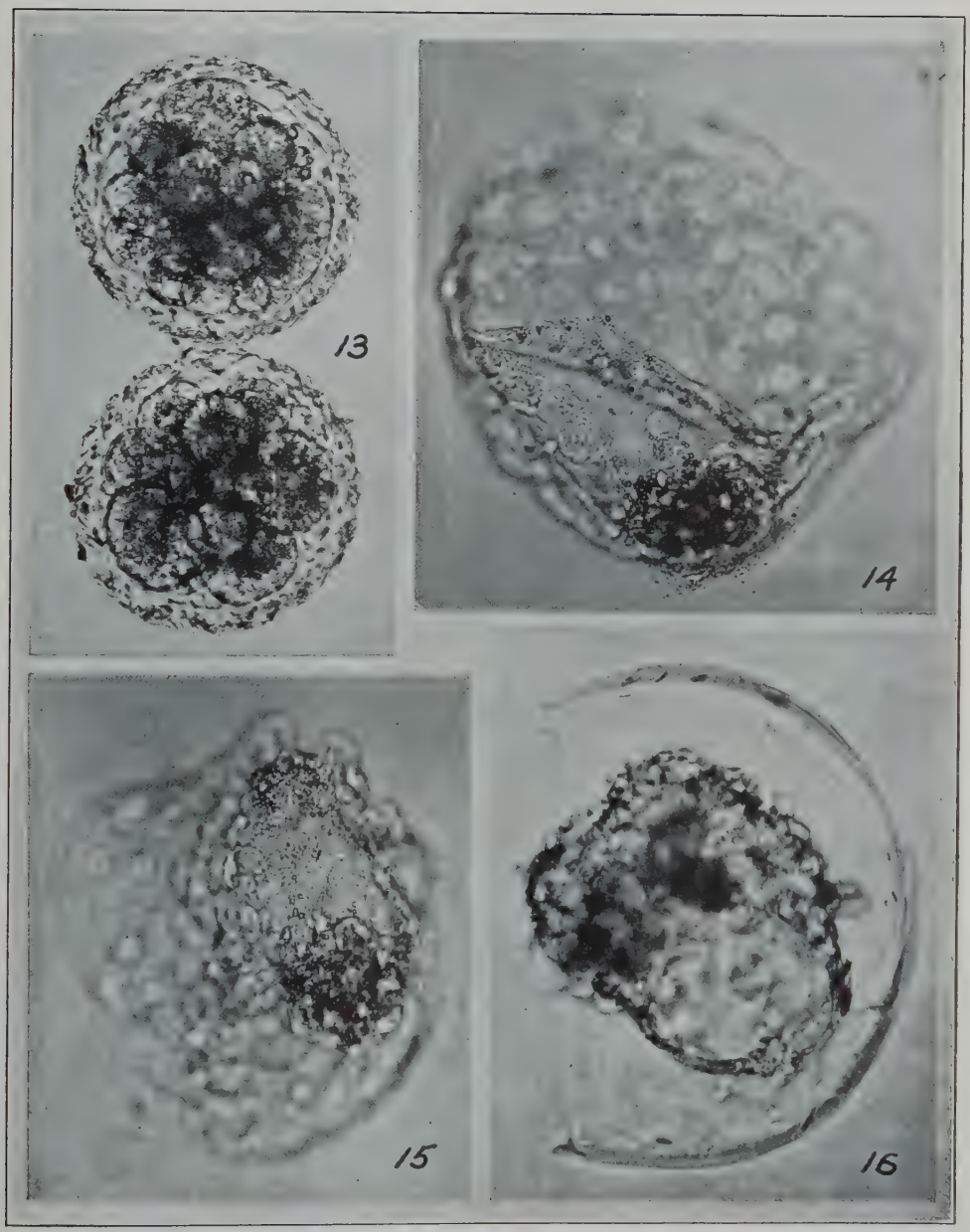
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PLATE 4

EXPLANATION OF FIGURES

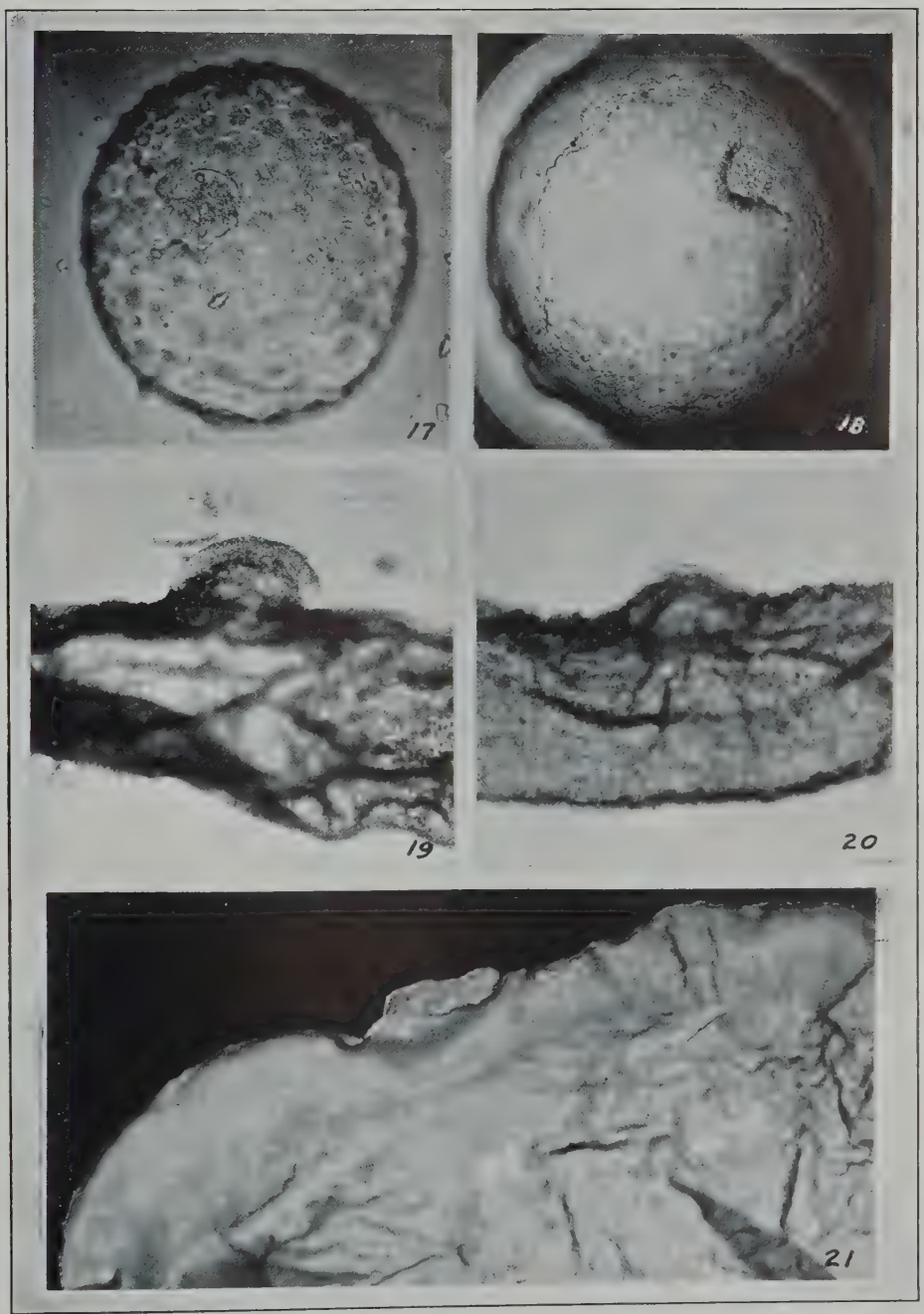
17 Trophoblast stage, 8 days 17½ hours post-coitum. Note large flattened trophoblast cells and inner cell mass. × 122.

18 Blastocyst, shortly after implantation, 10 days 19 hours post-coitum. Note change in cell type, as contrasted to vesicle shown in figure 17, and chorion commencing to elongate. × 50.

19 Blastocyst, 11 days 23¼ hours post-coitum. Note elongated chorion. × 43.

20 Blastocyst, 12 days 13¼ hours post-coitum. × 47.

21 Germ-disc, 13 days 3 hours post-coitum, showing primitive streak. Note tremendous enlargement of chorion. × 16.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STUDIES OF THE REACTION BETWEEN METHYLENE BLUE AND LIVING CELLS AND TISSUES IN THE TRANSPARENT MOAT CHAMBER INTRODUCED INTO THE RABBIT'S EAR [1, 2, 3]

RICHARD G. ABELL

Laboratory of Anatomy, University of Pennsylvania School of Medicine

TWO FIGURES

One of the most fundamental fields in morphology and physiology is that which involves the peripheral blood capillary, the various cells and tissues to which it supplies blood, and the intercellular material which may intervene between the two. In addition to the specific activity of the cells and the constant interchange of substances between them and the blood stream, this field includes the problem of the life story of the cell—its growth, maintenance, and death.

Curiously enough, comparatively little is known of the character of the intercellular substance and its modifications during varying phases of physiological activity. As a corollary our knowledge is equally meager regarding the effect which variations in the intercellular material may have upon the life story of the cell.

[1]This work was aided by a grant made to the Department of Anatomy at the University of Pennsylvania by the Rockefeller Foundation, under which the author held a fellowship. The assistance of a John Lockwood Memorial Traveling Fellowship from Swarthmore College, which was allotted to the author for the session 1932–1933, is also gratefully acknowledged.

[2]Brief reference to some of the results obtained in this study was made by Abell and Clark ('33) at the 1933 Meeting of the American Association of Anatomists.

[3]A dissertation in Anatomy presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfilment of the requirements for the degree of doctor of philosophy.

Certainly an important part of this field lies in the intercellular material, for it is this which forms the surrounding medium of the cell. The clearness with which the new growth of blood and lymphatic capillaries, the formation of arteries and veins, the inward migration of fibroblasts, the activity of macrophages, and the development and behavior of several other types of cells and tissues can be observed in transparent chambers inserted into the rabbit's ear, suggested the possibility of observing with the microscope the effect upon such structures of experimental changes in the character of the intercellular substance.

With the purpose of making such studies possible, a type of transparent chamber, known as the moat chamber, was developed by Abell and Clark ('32). This chamber permits the introduction of chemical substances into the intercellular region in a way that does not disturb mechanically the cells and tissues. Since the chamber is transparent, and the layer of cells and vascular tissues within it very thin, microscopic studies can be made of the effect upon the cells and tissues of such chemical modifications of the intercellular substance. Solutions installed within moat chambers can be subsequently removed and quantitative information secured concerning materials taken from and added to them by the living cells and tissues.

Having demonstrated the innocuous character of the installation of Ringer-Locke's solution into moat chambers, methylene blue was chosen for the first chemical studies. Several properties of methylene blue combine to make it particularly well suited for use in such initial experiments. In the first place, many cells stain vitally with methylene blue when in contact with solutions which are sufficiently dilute to be non-toxic. Consequently, under appropriate conditions, the distribution of methylene blue throughout the tissues can be observed following the introduction of non-injurious solutions of this dye into moat chambers. Information can thus be secured of the distance to which methylene blue is able to diffuse through vascular tissue from solutions of known concentration. In the second place, the colorimetric

method for determining the concentration of methylene blue can be used in analyzing solutions withdrawn from moat chambers, following exposure to vascular tissue. In this way the rate of removal of methylene blue by vascular tissue can be determined. In the third place, the ability of methylene blue to exist in a reduced, colorless form, as well as in the oxidized, blue condition, permits observations, in moat chambers, concerning the supply of oxygen by the blood to the tissues.

The present report describes experiments in which the removal of methylene blue by living capillaries and the effect of this dye upon a thin layer of cells and vascular tissue were studied in moat chambers. Data have been secured concerning oxygen diffusion from the peripheral blood capillary, the efficiency of blood capillaries in preventing the general diffusion of methylene blue through the tissue, the rate of removal of methylene blue by the vessels, the kind of stained cells, and the reactions of blood capillary endothelium to various concentrations of methylene blue.

For the opportunity of making these studies, and for many helpful suggestions concerning them, it is a pleasure to express my gratitude to Dr. Eliot R. Clark.

MATERIAL AND METHOD

1. Construction, installation, and care of moat chambers

Details concerning the construction, installation, and care of moat chambers may be obtained from the original description of the chamber by Abell and Clark ('32). Briefly, such a chamber, oval in shape, and composed principally of glass and mica, contains a very thin space called a 'bay,' which communicates at one end, termed the proximal end, with two entrance holes, and at the other end with a reservoir, or 'moat.' Chemical solutions may be placed in the moat, and subsequently removed from it, through two permanently installed access cannulae. Following the insertion of the chamber into a hole cut through the ear of a rabbit, living

tissue, continuous with the subcutaneous tissue of the ear, grows into the bay through the entrance holes. Since the bay, at its distal end, opens directly into the moat, chemical solutions introduced into the moat are free to pass into the bay and there to interact with the living cells and the tissue normally present during and following the vascularization of the chamber (compare figs. 1 and 2). Since moat chambers remain comparatively stable for many months (over a year in one case), and since the volume of the moat is very large compared to that of the tissue in the bay it is possible to conduct experiments which last any desired length of time up to a year or longer.

2. Injections of methylene blue solutions into the moat

a. Injections for which both cannulae were open. In these experiments, methylene blue solutions varying in concentration from 0.02 per cent to 0.05 per cent (1–5000 to 1–2000) were injected into moat chambers, sealed within them, and left for intervals varying in length from several hours to several days. They were then withdrawn, sterile Ringer-Locke's solution being injected into and sealed within the moat at this time. Daily records prepared with the help of a Leitz 'drawing eyepiece,' were made for several days preceding the injection of the methylene blue solution and throughout the experimental period. Photographs were taken to supplement these records, and the tissue was examined for several hours each day with the microscope. For some of these studies the oil immersion lens was used.

In every instance but one, the dye was injected into chambers in which the tissue had not yet grown all the way to the moat, so that before reaching the vessels, it had to diffuse through a region of the bay, between the tissue and the moat, which contained only tissue fluid, emigrated white blood cells, individual fibroblasts, strands of fibrin, cellular debris, and in some cases a few red blood cells.

The methylene blue solutions were prepared by the addition of dry material, secured from Grübler & Co., rectif. nach Ehrlich, to sterile Ringer-Locke's solution. For the technic of injecting these solutions into the moat, sealing them within it, and later removing them, reference should be made to the paper by Abell and Clark ('32) in which these manipulative procedures are described in detail. It is worth noting, however, that at the present time 70 per cent alcohol is used instead of hexylresorcinol for sterilizing the ends of the access cannulae, following their exposure. The alcohol is applied to the ends of the cannulae with a small cotton probe, before removal of the silver plugs, and is not dropped upon the open ends of the cannulae as was previously done with hexylresorcinol. Before the silver plugs are taken out of the cannulae, the alcohol is removed with a small sterile sponge, and the the surfaces of the cannulae allowed to dry completely by evaporation of alcohol. In this way any danger of diluting the contents of the moat with the disinfecting solution is removed. Two Luer syringes with the ends of the needles ground cone-shaped are used each time the moat is opened. With one syringe the moat contents are removed; with the second the moat is filled with the experimental solution, or with Ringer-Locke's solution. Luer syringes sterilized by dry heat are used.

b. Injections for part of which only one cannula was open. In one of the types of experiment to be described, it was necessary to inject the methylene blue solution into the moat with sufficient pressure to lift the top of the chamber slightly above the tissue in the bay. This was done in order to make certain that some of the methylene blue solution reached directly the upper surface of all of the tissue in the bay. When such an injection was to be made, the moat was filled with a solution of methylene blue, and a sterile silver plug placed in one of the cannulae. The tip of the syringe containing the methylene blue solution was then inserted in the open end of the other cannula, and a small additional quantity of methylene blue forced into the moat. Considerable pressure is neces-

sary to accomplish this, and unless some method is used to anchor the tissue in position, it is forced out of the bay. Such a method is the production of masses of precipitate at the periphery of the tissue. This can be accomplished by injecting into the moat a phosphate buffer prepared by adding 39.50 cc. of 0.2M NaOH to 50 cc. of 0.2M KH_2PO_4, sterilized by boiling. A barrier of highly insoluble material will be precipitated in the bay just in front of the most advanced capillaries, 24 to 48 hours following the injection of this solution into the moat. Tissue subsequently grows around these precipitated masses, and is held in the bay by them.

3. Experimental oxygenation of the peripheral cells and tissue in the bay

In order to determine the relation of the oxygen concentration to the distribution of stained cells in the bay between the most advanced capillary and the moat, oxygen was furnished to the cells in this region experimentally. In order to do this, oxygen from a tank containing pure compressed oxygen was passed through the moat, following the removal of the moat contents. Oxygen passed through the moat in this way is absorbed by the fluid in the bay, and readily diffuses to the region of living cells and tissue.

OBSERVATIONS

1. Distribution of stained cells following injections of methylene blue solutions into the moat

a. Distribution following injections during which both cannulae were open. The observations to be reported in this section were made with chambers in which the living, vascular tissue was separated from the moat by a distance which varied in different chambers from approximately 0.50 to 0.75 mm. At the time of the injections, the tissue was growing toward the moat, and microscopic observations made before the insertion of the dye showed the vessels to be normal in behavior and appearance. The distribution of stained cells, based upon a study of five moat chambers, was as follows.

Two to 4 hours after the injection of a 0.05 or a 0.02 per cent solution of methylene blue into the moat, the glass bottom of the bay close to the moat, the masses of leucocytes present in this region, and everything in the bay for a distance of a few micra from the edge of the moat, stain a diffuse, pale greenish-blue. During the next few hours this pale, diffuse color may advance slowly toward the vessels, but no cases have been observed in which the region of such diffuse staining adjacent to the moat increased in width to more than approximately half a millimeter. Six hours following the injection of the dye into the moat, though considerably longer in some cases, living cells which contain pale blue granules can be seen just peripheral but close to the most advanced blood capillary. Twelve to 15 hours later, many leucocytes and a few other types of cells to be subsequently described, contain brilliant blue granules. These stained cells are not distributed evenly throughout the bay, but are located in a narrow zone close to the most peripheral blood capillary. No stained granules have been observed in cells more peripheral to the most advanced capillary than 293 μ, nor, with few exceptions, have any blue granules been seen in cells more proximal to this capillary than 200 μ. Between the region of stained cells peripheral but close to the most advanced capillary, and the diffuse greenish-blue area adjacent to the moat, is a portion of the bay which shows no trace of blue color, in spite of the fact that it contains many living cells, and that methylene blue must diffuse through it in order to reach and to stain the cells close to the peripheral capillary. Two distinct regions of stained material, separated by a third region totally lacking blue color, can thus be observed subsequent to the injection of a 0.02 or a 0.05 per cent solution of methylene blue into the moat. These three regions are shown in figure 1.

There is some variation in different chambers of the distance in front of the most advanced capillary to which living cells stain vitally with methylene blue. In one chamber the most peripheral of such cells was 125 μ beyond the distal capillary—in another chamber 215 μ.

Microscopic examination of the vascular tissue between the most peripheral capillary and the entrance holes shows that as one passes toward the entrance holes the number of intensely stained cells decreases. In some instances there were no deeply stained cells proximal to the most advanced capillary. When such cells were present, their general location was in the region between the distal capillary and the one just proximal to it. It was only comparatively rarely that deeply stained cells were found in the tissue proximal to the second most advanced capillary. Although fairly large numbers of stained cells could be seen for a short distance in the tissue proximal to the second most advanced capillary, they generally contained pale blue rather than intense blue granules.

Not only does the number of intensely stained cells decrease as one passes from the peripheral capillary toward the entrance holes, but the actual number of stained cells decreases as well. At a greater distance proximal to the most advanced capillary than approximately 200 μ the number of stained cells present is very small, and all of them are located at a distance not greater than about 52 μ from venules and veins which form the principal collecting channels for blood from the periphery. Between these vessels the tissue is richly supplied with capillaries, yet contains no stained cells.

Fig. 1 A 'drawing eyepiece' record, showing the distribution of stained cells in a portion of the bay of chamber 2/23/33L, 24 hours following the introduction of a 0.02 per cent solution of methylene blue into the moat. All of the cells shown contained blue granules. Unstained cells are not indicated. The bay opens into the moat at the line BB. The dark region above BB represents methylene blue in a small portion of the moat. The peripheral capillary is located just proximal to 3_A. Sprouts were present on its distal side, but are omitted from this drawing. 1, region of diffuse greenish-blue staining in the bay adjacent to the moat; 2, the colorless region; many living cells were present throughout this region, but did not contain any blue granules; 3, region between the distal capillary and the moat in which many of the cells stained vitally with methylene blue; 3_A, that part of region 3 which was relatively free from debris and fibrin; 3_B, region proximal to the distal capillary in which cells showed concentration of methylene blue. Proximal to 4, only a few cells contained granules stained with methylene blue, and all were located close to venules and veins collecting blood from the peripheral capillary. The irregular line near the left margin of the drawing is the side of the bay. The portion of the bay included in this drawing is indicated in figure 2. × 89.4.

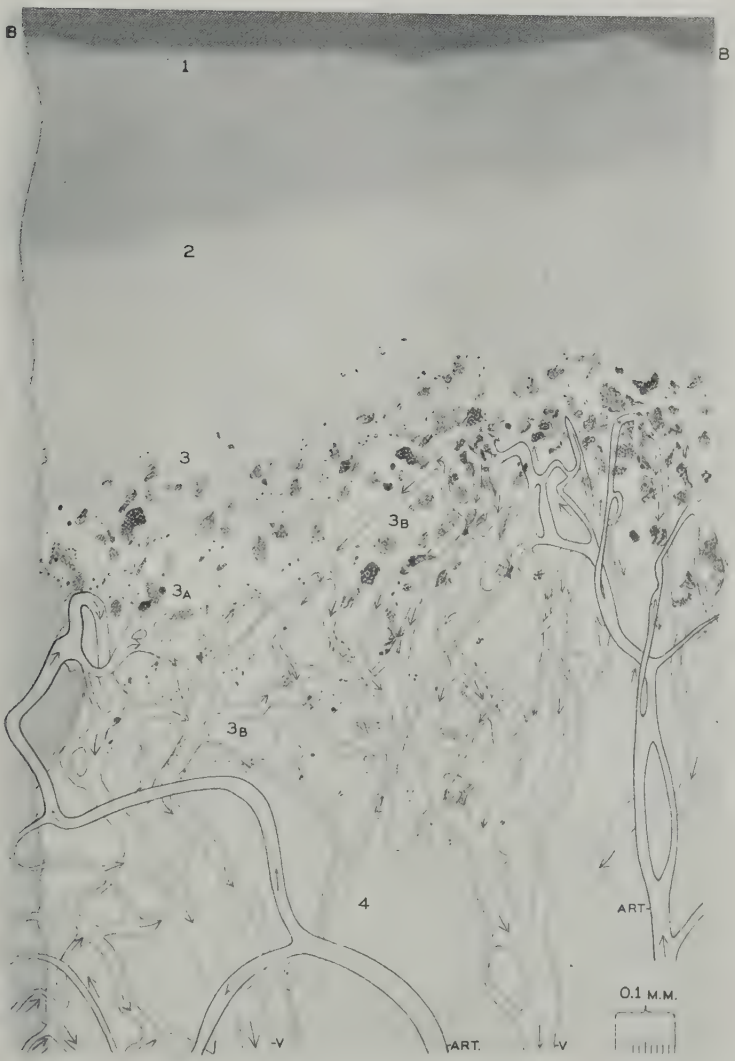

Figure 1

b. The effect of experimental oxygenation upon the distribution of stained cells between the most advanced capillary and the moat. A 0.05 per cent solution of methylene blue was injected into and sealed within the moat of chamber 1/23/32R on November 2, 1932. In this chamber the vessels had grown all the way into the moat, forming a narrow layer of living tissue in the moat at the end of the bay. The vessels of this narrow layer of tissue were injured by the methylene blue solution injected into the moat, so that the circulation through them was stopped. This checked the circulation in the vessels in the bay for a distance of approximately 0.68 mm. from the moat. The most peripheral part of the most advanced capillary containing actively circulating blood three days following the injection of the methylene blue solution was 0.68 mm. from the moat, and at this time a drawing eyepiece record showing the distribution of vitally stained cells in front of it was made. It was observed that, as in the case of the distribution of stained cells between the peripheral capillary and the moat in other chambers, the cells which showed concentration of dye were located in a narrow zone adjacent to the most peripheral capillary which contained actively circulating blood, and that no cells more than 215 μ in front of this capillary contained blue granules. Between the most peripheral of the stained cells and the moat, no trace of methylene blue could be observed, the pale, greenish-blue, diffusely stained region that can usually be seen adjacent to the moat being absent in this case. Following the completion of the drawing eyepiece record, the methylene blue solution was withdrawn, and oxygen passed through the moat. The living cells and tissues were watched continuously with the microscope during and following the passage of oxygen through the moat, and records of changes in the distribution of stained cells were made with the help of a drawing ocular. Details of the experiment are contained in the following protocol:

November 5, 1932, P.M.

4.09. Methylene blue removed from the moat.

410–4.13. Oxygen passed through the moat.

4.10–4.11. Material in the bay adjacent to the moat stained a diffuse greenish-blue for a distance which varied in different parts of the bay adjacent to the moat from approximately 160 to 225 μ. The typical colorless region between this greenish-blue stained area and the most peripheral of the stained cells close to the most advanced capillary could be clearly seen at this time.

4.20. Pale blue granules began to appear in some of the cells located in the originally colorless region, at its peripheral edge.

4.25. Stained granules appeared in more cells near the periphery of the region which had previously been colorless, and the depth of color deepened somewhat in those cells which first showed blue granules following the passage of oxygen through the moat.

4.27–4.30. Oxygen passed through the moat. The staining of cells gradually advanced toward the vessels, and those in which the blue color first appeared became more deeply stained.

5.00–5.45. Oxygen passed through the moat. The color progressively deepened in the newly stained cells, until it became as brilliant as in those close to the vessels which were stained before administration of oxygen was begun. The colorless region separating the newly stained cells from those close to the vessels gradually became narrower. At 5.45 the entire region between the greenish-blue diffusely stained area adjacent to the moat, and the region of original staining close to the most advanced capillary, was almost as completely filled with cells stained a brilliant blue as was the narrow zone close to the peripheral capillary which contained stained cells before oxygen was passed through the moat.

6.00. Ringer-Locke's solution injected into and sealed within the moat.

November 7, 1932, P.M.

1.45. The chamber was studied with the microscope. At this time the greenish-blue, diffusely stained region close to the moat was still present, and had not changed in appearance nor receded toward the moat. The cells in which blue granules appeared following passage of oxygen through the moat showed no trace of dye, but the cells in the original zone of staining close to the peripheral capillary contained brilliant blue granules.

c. Distribution of stained cells in the tissue proximal to the most advanced capillary, following injections of methylene blue during part of which only one cannula was open. Previous to the time of injection of methylene blue solutions into moat chambers, it had been supposed that after such injections the dye, upon reaching the peripheral margin of the living tissue, would gradually diffuse proximally through this tissue until it reached the entrance holes, at which time stained cells would be present throughout the entire extent of tissue in the bay. This did not turn out to be the case, however, the stained cells in the living tissue proximal to the most advanced capillary being limited in distribution to a narrow zone close to the peripheral capillary and to the principal vessels collecting blood from the periphery. It seemed desirable to determine whether this localization of stained cells was due to removal of the dye by the most advanced capillaries, which thus prevented it from reaching the proximal tissue, except by diffusion from the vessels which collected the blood from the periphery, or whether methylene blue actually did diffuse past the most advanced capillaries, but could not be seen in the more proximal tissue because of reduction. In order to find out which of these alternatives was the correct one, it was necessary to make certain that some of the methylene blue solution reached all of the tissue in the bay. This was accomplished by injecting the methylene blue solution into the moat with sufficient force to lift the mica top of the bay slightly above the tissue, thus permitting some of the dye to come in direct contact with the upper surface of all of the tissue in the bay. Twenty minutes following such an injection of 0.05 per cent solution of methylene blue into chamber 2/17/32L, in which the peripheral edge of the tissue was anchored by masses of precipitate, produced by a temporary installation in the moat of a phosphate buffer, many vitally stained cells could be seen throughout the entire extent of tissue in the bay. Large numbers of cells were stained in the proximal tissue all the way to the entrance holes, and, although most of these cells were located close to

blood vessels, there was no restriction of their distribution to the zone adjacent to the peripheral capillary or to the capillaries, venules, and veins draining blood from the periphery.

2. Description and classification of stained cells.

Many of the stained cells could be identified by their size and behavior as macrophages. A few of the adventitial cells, most of which were located along the more peripheral capillaries, but a small number of which were present in the more proximal tissue close to venules and veins collecting blood from the periphery, contained relatively large, pale, round, homogeneously stained structures. Definite, round, homogeneously stained structures were likewise observed in some, but not all, of the endothelial cells of the peripheral capillaries, and in a rather smaller proportion of the endothelial cells of the venules and veins draining blood from the periphery. Concentration of dye by macrophages was relatively lasting, in contrast to the temporary character of staining of adventitial and endothelial cells.

Beside the previously mentioned cells which could be definitely identified, many cells containing stained granules arranged in more or less regular rows were seen. Many deeply stained cells, smaller than macrophages, but probably leucocytes, were observed in the region peripheral to the most advanced capillary. In several instances leucocytes containing blue granules were observed within blood capillary sprouts and within blood capillaries containing actively circulating blood.

3. The rate of removal of methylene blue by the blood capillaries

Since the moat, as well as the bay, has a glass bottom and a mica top, marked variations in the depth of color of a methylene blue solution within it can be detected with the naked eye. During the 12 days that a 0.020 per cent solution of methylene blue was left in the moat of chamber 2 23 33L, daily

macroscopic observations showed that its color gradually became lighter. On the twelfth day, the color of the methylene blue solution within the moat was decidedly lighter than it had been when the solution was first placed in the moat.

In one case an attempt was made to determine colorimetrically the exact amount of methylene blue absorbed from the moat. Twelve days after the 0.020 per cent solution of methylene blue was injected into the moat of chamber 2/23/33L, a chamber which did not contain any lymphatic vessels, the moat was opened, its contents removed, and the concentration of methylene blue determined by comparison in a Spencer Duboscq colorimeter with a standard methylene blue solution. Two series of readings were made, the first immediately following the removal of the solution from the moat, the second 40 minutes later. A comparison of the results secured from these two series showed that the methylene blue solution did not change in color during this time, and that during the 12 days that the 0.02 per cent (1–5000) solution of methylene blue was left in the moat it decreased in concentration from 0.020 per cent to 0.003 per cent. Consequently, 85 per cent of the methylene blue present in the moat at the time the 0.020 per cent solution was sealed within it was removed in 12 days. Figure 2 is a photomicrograph of vessels and other structures in the bay of chamber 2/23/33L.

4. Removal of methylene blue by the lymphatics

Chamber 2/23/33L contained no lymphatic vessels, so that none of the methylene blue removed from the moat in this chamber could be attributed to lymphatic participation. A 0.05 per cent (1–2000) solution of methylene blue has been injected, however, into three chambers which contained both blood and lymphatic capillaries. In none of them did any of the leucocytes within the lymphatics show concentration of the dye, nor were any blue stained structures observed within cells which formed the walls of the lymphatics, or in cells near lymphatics. In every instance the lymphatics ended at least 200 μ behind the most advanced blood capillaries,

which thus intervened between them and the source of the dye. The distribution of stained cells in chambers containing both lymphatic and blood vessels was similar to their distribution in chambers that contained only blood vessels. There is thus no evidence from these studies to show that the lymphatics played any part in removal of the dye.

Fig. 2 A photomicrograph, showing blood vessels and other structures in the bay of chamber 2/23/33L 8 days following the installation of a 0.02 per cent solution of methylene blue into the moat. Brackets E and E_1 are placed below the edges of the two entrance holes through which the new tissue invaded the bay. The bay extends from the line D to the line BB. The arrows pointing upward are placed next arterioles, A; those pointing downward next veins, V. The portion of the bay shown at higher magnification in figure 1 is that included within the rectangle at the upper left corner. The legends for 1, 2, 3, 3_A, 3_B and 4 are identical with those given for the same designations in figure 1. × 17.5.

5. Reaction of blood capillary endothelium to methylene blue.

The injection of a 0.5 per cent solution of zinc free[4] methylene blue into the moat of chamber 6/14/33R caused severe hemorrhage, within 30 minutes, of all of the vessels in the bay for a distance of 0.4 mm. The installation of 0.05 per cent solution of methylene blue into chamber 1/23/32R, destroyed blood capillaries present in the moat at the time of injection of the dye. A slight hemorrhage of the most advanced capillary loops in the bay of chamber 7/30/31L was produced by a 0.05 per cent solution of methylene blue, sealed within the moat and left overnight. In other instances the injection of a 0.05 per cent solution into the moat did not produce hemorrhage of any of the vessels in the bay. The injection of a 0.02 per cent solution of zinc free[4] methylene blue into the moat of chamber 2/23/33L destroyed one peripheral capillary loop, but not others adjacent to it. Staining of the nuclei of the endothelial cells of this particular capillary loop preceded its destruction. Why one capillary loop should have been destroyed but not others near it, is unknown.

In some cases sticking of leucocytes to the endothelium developed in the peripheral capillary and in the venules and veins collecting blood from the periphery, following the introduction of a 0.05 per cent solution of methlene blue into the moat. In other instances a methylene blue solution of the same concentration produced no such sticking. A 0.003 per cent solution of methylene blue present within the moat of chamber 2 23 33L produced no visible toxic effect upon the vessels in the bay.

[4]For analysis of a sample of the methylene blue used in this experiment I am indebted to Dr. John H. Muller, professor of chemistry in the Graduate and Towne Scientific Schools of the University of Pennsylvania.

DISCUSSION

The distribution of cells showing concentration of methylene blue in the region between the most advanced blood capillaries and the moat, and the presence, distal to such stained cells, of a portion of the bay in which no trace of blue color can be detected appears to be consistent with the view that as the methylene blue diffuses from the moat into the bay, it undergoes alternate reduction and oxidation. According to this conception, the methylene blue is changed to leucomethylene blue as it passes into the region of living cells removed from the blood capillaries by a distance greater than approximately 293 μ. When the leucomethylene blue enters the region of higher oxygen concentration close to the blood capillaries, it is oxidized within many of the cells, possibly also outside of them, to methylene blue.

That living tissue is able to reduce methylene blue has been demonstrated by the studies of Ehrlich (1885 and 1886), Arnstein (1887), Underhill and Closson ('05) and others. That reduced methylene blue actually was present in the colorless region of the bay, following the introduction of a solution of methylene blue into the moat, is demonstrated by the appearance of brilliant blue granules in many of the cells in this region, subsequent to experimental increase of the oxygen concentration.

The data indicate the presence of a gradient of oxygen concentration at the periphery of vascular tissue in moat chambers.

The present observation that a 0.05 per cent solution of methylene blue, introduced into moat chambers, does not diffuse quickly throughout the tissue in the bay, is interesting in connection with observations described by E. R. and E. L. Clark ('33) concerning the character of the intercellular substance. These investigators present evidence that in living subcutaneous tissue in 'round-table' chambers introduced into the rabbit's ear, free fluid is not present outside of the lymphatic and blood vessels. This appears also to be true of the vascularized tissue in moat chambers. Although the oscil-

lation of extravasated blood cells indicating free fluid can frequently be seen in the unvascularized area during the first few days of growth of new vessels and connective tissue into moat chambers, this movement ceases above and between the tissue cells with the progressive vascularization of the chamber. Bobbing back and forth of blood cells can sometimes be observed between the peripheral capillary and the moat even during later stages of ingrowth, but the material above the vascular tissue loses its fluid character. So also does that between the tissue cells. If free fluid were actually present outside of the blood and lymphatic vessels, or between the tissue and the mica cover of the bay; it appears likely that more general diffusion of methylene blue would occur.

The experiments of Ehrlich (1885 and 1886) show that tissues containing reduced methylene blue acquire a blue color upon exposure to air, due to oxidation of the leucomethylene blue into the blue product. Underhill and Closson ('05) demonstrated the excretion of methylene blue in the urine in the form of leucomethylene blue (salt) as well as in the form of methylene blue, and showed that such methylene blue urine gradually assumes a deeper color upon exposure to air for 25 to 30 minutes. If the urine is made alkaline, the change in color occurs at once, due to formation and rapid oxidation of leucomethylene blue (base).

There is a possibility that the gradual change in depth of color of a methylene blue solution sealed within the moat, with free access to the blood vessels in the bay, may have been due to partial reduction of the methylene blue by substances diffusing into the moat from the living tissue in the bay. Assuming that this may have been the case, it appears evident that no methylene blue in the solution removed from the moat was present in the reduced form at the time its concentration was colorimetrically determined, since no change in color of the solution occurred during exposure to the air for 40 minutes. This length of time should have been sufficient to cause a change in depth of color even had the reduced methylene blue been present in the form of the salt.

The colorimetric determination of the concentration of methylene blue in the solution removed from the moat 12 days following its injection into the chamber is believed, therefore, to show, within the limits of experimental error of the method, the actual concentration of methylene blue in the solution at this time, and consequently to demonstrate that a solution of methylene blue, injected into and sealed within the moat, with free access to living blood capillaries in the bay, undergoes decrease in concentration. Under the conditions present during this experiment, 85 per cent of the methylene blue present at the time of injection of the methylene blue solution into the moat was removed in 12 days.

Staining of granules in endothelial cells of the peripheral capillaries and of the venules and veins collecting blood from the periphery, and the appearance of blue granules in adventitial cells and macrophages located in such positions in the proximal tissue that methylene blue was able to reach them only by rediffusion out of the vessels collecting blood from the periphery, show that methylene blue actually entered the peripheral blood capillaries in the bay. The distribution of stained cells in the tissue proximal to the most peripheral capillary indicates that most of the methylene blue was removed by way of the two most advanced rows of capillaries, the greater part probably by way of the capillary closest to the source of the dye, that is, the most peripheral capillary.

Direct passage of methylene blue through the walls of the blood vessels following injection of solutions of this dye into serous cavities of the dog was reported by Starling and Tubby (1894). Landis ('26) observed that certain dyes (including methylene blue) pass through the walls of the venous capillary almost as easily as through simple endothelium, while Clark, Clark and Williams ('34) demonstrated that methylene blue, following intravenous injection into vessels of the rabbit's ear, is able to pass through the walls of veins as well as through those of the capillaries. In this connection the experiments of Shipley and Cunningham ('16), who reported the active absorption of potassium ferrocyanide and iron ammonium

citrate through the walls of arteries and veins of the cat's omentum, as well as through those of the smaller vessels, are of interest. The present work indicates the rediffusion of methylene blue through the walls of the venules and veins collecting blood from the periphery, as well as its passage through the walls of the capillaries.

The absence of any stained structures in the lymphatics, in those chambers which contained lymphatics as well as blood vessels, affords additional evidence that the last two rows of blood capillaries removed the methylene blue, allowing none of the dye to reach the lymphatics. That leucocytes within the tips of the lymphatics would have stained blue had the dye reached them is indicated by the observations of Clark, Clark, and Williams ('34), who reported the appearance of blue stained leucocytes in a lymphatic in a round-table chamber inserted into the rabbit's ear, following direct injection of a solution of methylene blue into an adjacent vein. Had the lymphatics extended to the periphery of the tissue in the bay, it appears likely that some of the leucocytes within them would have stained blue.

The apparent absence of removal of methylene blue by the lymphatics in the present experiments is in accord with the previous observations of E. R. and E. L. Clark ('31) that in round-table chambers the movement of lymph in newly formed lymphatic vessels is very sluggish—frequently the same leucocytes or erythrocytes remaining in a capillary for several days, or even weeks. That the presence of lymphatic vessels is not necessary for the cleaning up of extra-vascular debris during ingrowth of new tissue into round-table chambers was demonstrated by E. R. and E. L. Clark ('33).

SUMMARY

1. Following the introduction of solutions of methylene blue into moat chambers, many cells which contain blue granules can be observed in the bay. With few exceptions, such cells are located in a zone extending from a line approximately 293 μ distal to the terminal capillary loops to a line about 200 μ proximal to them.

2. Although, under the conditions normally present in moat chambers, no cells that are located more than 293 μ in advance of the peripheral blood capillary loops have been observed to stain vitally with methylene blue, brilliant blue granules suddenly appear in many such cells when the oxygen concentration is experimentally increased, indicating a previous concentration of methylene blue in such cells in the reduced form.

3. The data indicate that a gradient of oxygen tension is present at the periphery of vascular tissue in moat chambers.

4. Concentration of methylene blue by macrophages is relatively lasting, in contrast to the temporary character of staining of adventitial and endothelial cells.

5. General diffusion of methylene blue through the tissue in the bay did not occur, evidently because of removal of the dye by the blood circulating through the peripheral capillaries.

6. Quantitative data are presented showing the rate at which the methylene blue was removed by the blood capillaries.

7. Blood capillaries were rapidly destroyed by a 0.5 per cent solution of zinc-free methylene blue installed in the moat. They showed no appreciable reaction to a 0.003 per cent solution, while their reaction to solutions of 0.02 to 0.05 per cent varied from a mild stickiness toward leucocytes to an absence of any visible endothelial change.

8. There is no evidence to show that lymphatics, when present, played any active part in removal of the methylene blue.

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MICROSCOPIC OBSERVATIONS OF STRUCTURAL CHANGES IN THE ADRENAL GLAND OF THE LIVING FROG UNDER EXPERIMENTAL CONDITIONS

EDWARD SINGER AND R. L. ZWEMER

Department of Anatomy, College of Physicians and Surgeons, Columbia University

The possibility of visually correlating the function of a gland of internal secretion with its changing structure opens up an interesting field for study. It was first suggested by Vonwiller and Zulzer in 1927, in a paper on the kidney, where they briefly describe the living adrenal gland.

The present observations on the adrenal glands of living frogs enable us to report a definite continuity in their dynamic morphology.

The technique of observation has been described in detail elsewhere (Singer, '32). The fluorescence microscope and Busch Univertor were used, which permitted the use of reflected light and dark-field illumination with visible light, and of filtered ultraviolet light, which induced fluorescence. This microscope used in connection with specially devised water immersion lenses made it possible for us to observe the adrenal gland with high magnifications and for long periods of time, the gland being kept constantly and evenly moist with gently-flowing Ringer solution.

The frogs were immobilized by pithing, curare or ether, but prolonged observation of controls showed that the least change in adrenal structure occurred during the light ether anesthesia. The appearance of the living adrenals in more than fifty frogs was compared with fixed, sectioned and stained tissues from the same and similarly treated animals. Both

paraffin and gelatin-freezing (Zwemer, '33) techniques were used.

In the adrenal gland of the normal frog, with white reflected light and low power, the cell groups appear as irregularly rounded or elongated oval, yellow islands surrounded on all sides by bright red, moving blood streams. No cell boundaries or nuclei are seen.

With higher magnification ($\times$ 480) an indistinct cellular demarcation and a few nuclei become visible. The cells are filled with fine round yellow or yellow-brown granules. The nuclei, when seen, are nearly round and have a clear gray-yellow color. The larger, fast-moving blood streams are adjacent to the long diameters of the cell groups. The smaller cross-anastomoses are slower and may either stop or even reverse their flow. Under high power the red blood cells resemble bright shining plates of yellow gold.

Phagocytic leucocytes, made more visible in some of the cases by ingested blue dye, drift by with the current, or migrate slowly along the capillary walls. They settle down from time to time and then move on again. Fixed preparations reveal the constant presence of these cells in the adrenal gland. This is not strange when we remember that in the mammal the adrenal gland is generally cited as the location of part of the 'reticulo-endothelial' system.

The typical, yellow-granular normal cells frequently undergo a change which we have been unable to associate with any experimental procedure. The granules increase in size in all diameters until within 15 minutes they assume the appearance of oil globules. These are crowded together and fill the entire cytoplasm, obscuring the nucleus. The globules increase in size so rapidly that in half an hour no cell boundaries can be distinguished and the entire area of the changing section is covered by large globules interspersed with smaller globules of varying sizes. Adjacent cell groups frequently remain unchanged, and there is no change in the nearby kidney cells. This sporadic change from granules to globules in the adrenal cells could not be correlated with injections, exposure to light or extreme variations in blood circulation.

Filtered ultraviolet light, used after the injection of 1 cc. of a saturated solution of aesculine, reveals a network of fine brown fibers meshed in the fluorescent, brown cell-islands. This network is no longer visible when the globular change occurs. About 6 to 10 minutes after the injection of aesculine the margins of the cell clumps begin to appear brown, while the centers are still purple from the reflected light of the filter. Ten to 20 minutes later the entire adrenal gland is fluorescent and blood streams made visible by their bluish glow are seen weaving in and out among the brown cell-islands. No nuclei or cell boundaries are visible with aesculine, but 2 minutes after the injection of 1 cc. of 1:1000 solution of acriflavine the nuclei are fluorescent. This last named solution does not affect the cytoplasm.

Etherized frogs observed without injections show no specific changes in their adrenal glands. Even after hours of observation the gland cells of control frogs under ether anesthesia appear the same as when the abdomen was first opened, save for the spontaneous change previously described.

Pithing and curare, on the other hand, seem to increase the visibility of nuclei and cell boundaries. In pithed frogs the nuclei are darker than the cytoplasm in full reflected light, but become brilliant shining spots in the dark cytoplasm when half-shadow illumination is used. The cells appear smaller and crowded together. Curare shows the multangular cell boundaries and slightly ovoid nuclei with special clearness about 30 to 40 minutes after injection. Occasional empty spaces appear between the fine evenly granulated yellow cells. A subsequent return to more normal conditions is frequently observed.

Bichloride of mercury poisoning (1 cc. or 2 cc. of 1:1000 solution) is followed by a gradual change in the cytoplasmic structure. The pale yellow background begins to fade, and the yellow-brown granules enlarge until they appear as pale droplets or bubbles. At the end of half an hour many of the cell groups have a frothy, water-white appearance. Finally, the grayish nuclei become distinct and the cell boundaries

definitely outlined. At this stage the granules still present are either coarser and uneven in size and distribution, or are extremely fine, and the cytoplasm is very faint yellow or white, which gives the cell a watery appearance. A profile view of the growth of granules to bubbles reveals an extrusion phenomenon, the bubbles being projections into the lumina of the blood vessels. The increased visibility of the nuclei and cell boundaries is probably due to a further loss of substance from the cell.

These observations, when correlated with our studies of fixed tissues, indicate that the adrenal gland of the normal frog contains a yellow substance located in cells homologous to the corticoadrenal cells of mammals. This substance is stained by osmium tetroxide, sudan III and IV, and Nile blue sulphate. From its staining reactions, it does not appear to be a neutral fat. Some anisotrophic lipoid is in intimate relation with the stained-lipoid in the normal frog. Both decrease rapidly when the frogs are subjected to certain treatments. Examination of sectioned tissues shows that ether produces the least change in the lipoids; pithing and curare are intermediate; and mercuric chloride injections are followed by the greatest lipoid depletion.

SUMMARY

1. The adrenal gland of the living frog has been studied by reflected white and fluorescent light.

2. In the normal gland the cells are filled with small, evenly-distributed, yellow granules.

3. The granular condition can change to a globular one. This phenomenon is unassociated with the experimental procedure.

4. Fluorescent light reveals a fine network of fibers around the granular cells, which is not visible when the globular condition prevails.

5. Pithing, curare and bichloride of mercury induce changes in adrenal cell structure which are apparently due to a diminution of their lipoid content.

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ORIGIN OF THE DEEP AND CIRCUMFLEX FEMORAL GROUP OF ARTERIES [1]

GEORGE D. WILLIAMS, CHRISTOPHER H. MARTIN AND LANDON R. McINTIRE

Department of Anatomy, Washington University School of Medicine

ONE FIGURE

In 1930, the authors and others reported on the types of origin of the deep and circumflex femoral group of arteries in cadavers available to this department of anatomy. After publication, observations were continued on similar material by the authors of this paper with a view to supplementing the data already reported. The 166 arterial patterns of the 1930 material are distributed as follows:

	White male	*Negro male*
Right	55	29
Left	52	30

The distribution of the new data is:

	White male	*Negro male*	*White female*	*Negro female*
Right	59	54	13	30
Left	59	57	13	30

permitting a distribution of the total material (481 patterns) as follows:

	White male	*Negro male*	*White female*	*Negro female*
Right	114	83	13	30
Left	111	87	13	30

Seven types of patterns were described in the earlier paper; viz., 1, 1a, 2, 3, 4, 4a, and 5.[2]

[1] The authors wish to acknowledge aid given to this investigation by the Rockefeller Foundation.

[2] Figure 1. For the sake of brevity, numbers are here used to indicate types. The Barchow-Adachi terms have the following equivalents: truncus profundo-circumflexus perfectus = type 4; truncus profundo-circumflexus lateralis = type 2; truncus profundo-circumflexus medialis = type 3.

Type 4a is a minor variation of type 4, and varies from it only in that the descending artery arises directly from the deep femoral rather than from the lateral circumflex femoral artery. To simplify the classification, type 4a has been dropped in this paper. The availability of a greater number of observations suggested that types of patterns different from those previously described were to be expected. Three new types (1b, 1c,[3] and 6) were discovered. The nine types now recognized are discussed in consideration of series II and are shown diagrammatically in figure 1.

Consideration of the data here presented first poses the question as to whether the percentage incidence of types in the later series (series II) differs significantly from that in the earlier (series I). Table 1 shows these percentage distributions (omitting the new types of series II), together with their respective differences and the standard errors of those differences. The standard errors of the percentage differences were calculated according to the formula of Yule ('27, p. 269):

$$\text{Standard error squared of the difference between percentage 1 and percentage 2} = p_0 q_0 \left(\frac{1}{n_1} + \frac{1}{n_2} \right)$$

Table 1 shows no case in which the percentage difference exceeds by three times its standard error and only one case in which the difference is twice the error. This latter situation is due to the fact that type 5 is unrepresented on the right side of white males of series I; if but one case had occurred, the possibility of significant difference would disappear. It may be concluded from table 1 that the apparent differences in percentage incidence between series I and series II have arisen through the chances of simple sampling.

Since no significant differences appear between these two series in either white or negro males, the two samples in the case of each race were combined into one. The cases of types

[3] One case of type 1c was found. It is of particular interest in that it closely approximates the realization of the possibility of four separate branches from the femoral artery. The case is here interpreted as one of three branches because the deep and descending arteries arise from the femoral by a very short common stem.

1b, 1c and 6 of series II were added to the new combination, as well as all the female cases, making a total of 481 arterial patterns available for statistical study.

This paper considers three factors which may be correlated with variations in percentage distribution of types: side, sex,

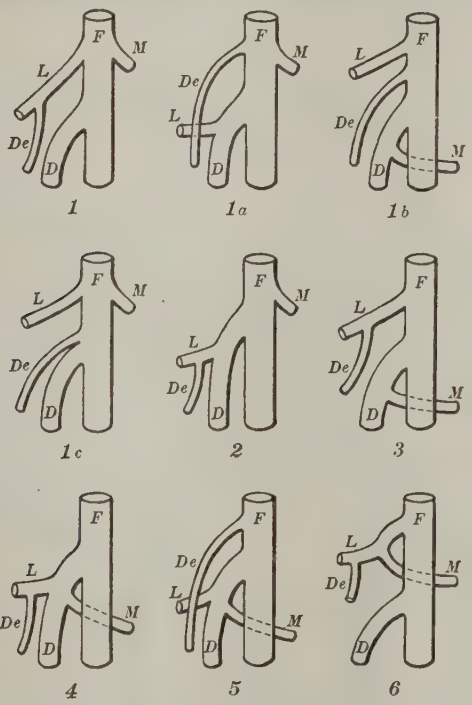

Fig. 1 Types of origin of the deep and circumflex femoral group of arteries. D, deep femoral; De, descending circumflex femoral; F, femoral; M, medial circumflex femoral; L, lateral circumflex femoral.

and race. Table 2, part I compares percentage incidence according to side, regardless of race in the two sexes. Table 2, part II, makes a similar comparison according to sex, regardless of side in each race. Neither set of data shows a case in which a difference is as much as twice its standard error; from which it follows that neither side nor sex is of

importance in the determination of incidence of the various types of origin of the deep and circumflex femoral group of arteries.

In table 2, part III (comparison of races, regardless of side for the two sexes), are found two differences, each of which exceeds its standard error by more than two times. The per-

TABLE 1

Percentage distribution of types of origin of the deep and circumflex femoral group of arteries

White males

	RIGHT			LEFT		
Type	Series I (55)	Series II (58)	Difference	Series I (52)	Series II (59)	Difference
4	49.1	48.3	0.8 ± 9.4	65.4	54.2	11.2 ± 9.3
2	27.3	20.7	6.6 ± 8.0	17.3	27.1	9.8 ± 7.9
3	14.5	13.8	0.7 ± 6.5	11.5	10.2	1.3 ± 5.9
5	0.0	8.6	8.6 ± 3.8[1]	3.8	5.1	1.3 ± 3.9
1	9.1	3.4	5.7 ± 4.5	1.9	3.4	1.5 ± 3.1
1a	0.0	5.2	5.2 ± 3.0	0.0	0.0	0.0

Negro males

	RIGHT			LEFT		
Type	Series I (29)	Series II (52)	Difference	Series I (30)	Series II (57)	Difference
4	58.6	55.8	2.8 ± 11.5	70.0	57.9	12.1 ± 10.9
2	10.3	19.2	8.9 ± 8.5	20.0	17.5	2.5 ± 8.7
3	20.7	21.2	0.5 ± 9.4	3.3	17.5	14.2 ± 7.5
5	0.0	1.9	1.9 ± 2.5	0.0	0.0	0.0
1	6.9	1.9	5.0 ± 4.4	3.3	7.0	3.7 ± 5.2
1a	3.4	0.0	3.4 ± 2.5	3.3	0.0	3.3 ± 2.3

[1] Difference twice its standard error.

centages on which the differences are based may be found in part II of the same table. Ten cases of type 5 occurred in 225 white males, while only one was found in 170 negro males. The table also shows that type 2 is considerably more frequent in white than in negro females, but the number of observations in each case is small. Turning to part IV of table 2, it is noted that with side and sex disregarded, the same

TABLE 2

Percentage distribution of types of origin of the deep and circumflex femoral group of arteries

I. Comparison of sides, regardless of race

	MALE			FEMALE		
Type	Right (197)	Left (198)	Difference	Right (43)	Left (43)	Difference
4	51.3	60.6	9.3 ± 5.0	58.1	53.5	4.6 ± 10.7
2	20.3	20.7	0.4	16.3	25.6	9.3 ± 8.8
3	16.8	11.6	5.2 ± 3.5	16.3	7.0	9.3 ± 6.9
5	3.0	2.5	0.5	0.0	0.0	0.0
1	4.1	4.0	0.1	7.0	7.0	0.0
6	1.0	0.0	1.0	2.3	2.3	0.0
1a	2.0	0.5	1.5	0.0	2.3	2.3
1b	1.0	0.0	1.0	0.0	2.3	2.3
1c	0.5	0.0	0.5	0.0	0.0	0.0

II. Comparison of sexes, regardless of side

	WHITE			NEGRO		
Type	Male (225)	Female (26)	Sex difference	Male (170)	Female (60)	Sex difference
4	53.8	42.3	11.5 ± 10.3	58.8	61.7	2.9
2	23.1	34.6	11.5 ± 8.9	17.1	15.0	2.1
3	12.4	15.4	3.0	16.5	10.0	6.5 ± 5.3
5	4.4	0.0	4.4	0.6	0.0	0.6
1	4.4	7.7	3.3	3.5	6.7	3.2
6	0.0	0.0	0.0	1.2	3.3	2.1
1a	1.3	0.0	1.3	1.2	1.7	0.5
1b	0.0	0.0	0.0	1.2	1.7	0.5
1c	0.4	0.0	0.4	0.0	0.0	0.0

III. Comparison of races, regardless of side

Type	MALE RACIAL DIFFERENCE	FEMALE RACIAL DIFFERENCE
4	5.0 ± 5.1	19.4 ± 11.7
2	6.0 ± 4.1	19.6 ± 9.6[1]
3	4.1 ± 3.6	5.4 ± 7.5
5	3.8 ± 1.7[1]	0.0
1	0.9	1.0
6	1.2	3.3
1a	0.1	1.7
1b	1.2	1.7
1c	0.4	0.0

IV. Comparison of races, regardless of side and sex

TYPE	WHITE (251)	NEGRO (230)	DIFFERENCE
4	52.6	59.6	7.0 ± 4.5
2	24.3	16.5	7.8 ± 3.7[1]
3	12.7	14.8	2.1
5	4.0	0.4	3.6 ± 1.4[1]
1	4.8	4.3	0.5
6	0.0	1.7	1.7
1a	1.2	1.3	0.1
1b	0.0	1.3	1.3
1c	0.4	0.0	0.4

[1] Difference twice its standard error.

types, 2 and 5, show greater incidence in whites than in negroes, with differences which are at least suggestive of statistical significance.

Adachi ('28) dealt with the problem with which we are concerned, and with his own data on Japanese presented comparative figures which are quoted in table 3.[4] Table 3 also includes the data of this paper.

Table 4 summarizes the racial differences in the percentage frequencies of table 3. Only the differences in types 4, 2, and 5 are listed, since all differences in the remaining types

TABLE 3

Percentage distribution of types of origin of the deep and circumflex femoral group of arteries

TYPE	JAPANESE—ADACHI (367)	NEGROES—THIS PAPER (230)	WHITES—THIS PAPER (251)	ENGLISH—QUAIN (378)	GERMANS—RUGE (50)	PRUSSIANS—AUBURTIN (100)	AUSTRIANS—SEB (200)	WHITES HERE QUOTED (979)	NEGROES AND JAPANESE HERE QUOTED (597)
4	63.2	59.6	52.6	51.3	52.0	55.0	62.0	54.2	61.8
2	15.0	16.5	24.3	18.5	38.0	25.0	20.5	22.1	15.6
3	14.4	14.8	12.7	15.9	8.0	15.0	13.0	14.0	14.6
5	2.2	0.4	4.0	8.2	0.0	0.0	0.0	4.2	1.5
1	3.5	4.3	4.8	2.9	0.0	4.0	3.5	3.9	3.9
6	0.8	1.7	0.0		2.0	1.0	1.0		1.2
1a	0.5	1.3	1.2	3.2	0.0	0.0	0.0	1.5	0.8
Other types	0.3	1.3	0.4					0.1	0.7

are insignificant. The whites of this paper appear to be similar in distribution of types to the sample representing the total of all whites quoted in table 3. The probably significant difference in types 2 and 5 between the whites and negroes of this paper has already been noted. Our white group compared with Adachi's Japanese shows another difference of possible significance in incidence of type 4, while the larger sample of whites compared with the Japanese gives a type 4 difference of three times its standard error which must be considered as significant. The total whites differ from our

[4] The percentages of Lipshutz on ninety-nine American whites and negroes have been omitted, because that author grouped whites and negroes into one series.

negroes by twice the error in incidence of type 5; our whites differ from our negroes by twice the error in incidence of types 5 and 2.

Since the American negroes and Japanese appear (in table 4) not to differ from each other by as much as twice the standard error in the incidence of any type, and since the whites of this series and of the total series do differ to that extent from each of the non-white groups, it seems logical to combine the negroes and Japanese into one series for purposes of comparison with the two white samples. Table 4

TABLE 4

Differences in percentage distribution of types of origin of the deep and circumflex femoral group of arteries. The standard error of each difference is shown

TYPE		TOTAL WHITES	NEGROES (THIS PAPER)	NEGROES PLUS JAPANESE	JAPANESE
Whites (this paper)	4	1.6 ± 3.5	7.0 ± 4.5	9.2 ± 3.7[1]	10.6 ± 4.0[1]
	2	2.2 ± 3.0	7.8 ± 3.7[1]	8.7 ± 2.9[2]	9.3 ± 3.2[1]
	5	0.8 ± 1.3	3.6 ± 1.4[1]	2.5 ± 1.1[1]	1.8 ± 1.4
Total whites	4		5.3 ± 3.6	7.6 ± 2.6[1]	9.0 ± 3.0[2]
	2		5.6 ± 3.0	6.5 ± 2.1[2]	7.1 ± 2.4[1]
	5		2.8 ± 1.2[1]	1.7 ± 0.7[1]	1.0 ± 1.0
Negroes (this paper)	4				3.6 ± 4.1
	2				1.5 ± 3.0
	5				1.8 ± 1.0

[1] Difference twice its standard error.

[2] Difference three times its standard error.

shows that each white series differs from the combined Japanese and negroes by twice the standard error in types 4 and 5, and by three times the error in type 2. The non-whites exhibit more frequently type 4; the whites show greater incidence of types 2 and 5.

Loth (1931), summarizing the work of many authors, states: that type 1 is the form common to many mammals, including the platyrrhine monkeys and some catarrhines; that type 2 is frequent in the cercopithecinae and most of the great apes; that the semnopithecinae often show type 3; but that type 4 is common in man and rare in other primates. (He makes no statement concerning type 5.) Loth further states:

Nous constatons une difference assez importante entre les Blancs et les Japonais. Rappelons que les formes primitives (*truncus profundo-circumflexus lateralis*) sont moins frequentes chez les Japonais tandis que le *truncus profundo-circumflexus perfectus,* qui est, comme nous nous l'avons dit plus haut une forme humaine, est au contraire plus frequent chez eux. En somme au point de vue des ramifications de l'artere femorale, les Japonais sont plus progressifs que les Blancs.

If the Japanese show fewer primitive (mammal- or ape-like) and more human types of branching than do the whites, the data here presented suggest that American negroes differ similarly from whites. In the absence of definite information concerning the occurrence of type 5 in apes or mammals, the authors suggest that type 5 is a primitive type just as is type 2. Types 5 and 2 (more frequent in whites than in non-whites) represent the opposite of the progressive tendency for the femoral artery to branch by means of a common stem as in type 4, which pattern is more common in American negroes and Japanese.

SUMMARY

Observations on the origin of the deep and circumflex femoral group of arteries in 481 thighs (of 251 whites and 230 American negroes) show no side or sex differences in incidence. Negroes exhibit less frequently than whites the truncus profundo-circumflexus lateralis and that type in which the descending artery, usually a branch of the lateral femoral circumflex, originates from the femoral. In the incidence of types, the negroes do not vary significantly from the Japanese. Japanese and negroes grouped together show fewer of the two phylogenetically primitive types just mentioned, and a greater proportion of the phylogenetically progressive truncus profundo-circumflexus perfectus.

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THE DISTRIBUTION AND DRAINAGE OF OMENTAL LYMPHATICS IN THE DOG AND CAT

PARKE H. SIMER

Department of Anatomy, College of Medicine, University of Illinois, Chicago, Illinois

FIVE FIGURES

In a recent paper ('34) the writer established the presence of omental lymphatics in the white rat and in man. The occurrence of similar channels in other species has long been an open question which has aroused great interest. This investigation affords a definite answer to the problem in so far as the dog and cat are concerned, and it demonstrates the paths of lymph drainage in these animals from the omental vessels to the thoracic duct.

REVIEW OF LITERATURE

Numerous investigators have studied the omentum of the dog. Robinson (1895) discussed its macroscopic anatomy and concluded that this organ is a great peritoneal protector against infectious diseases. A few years later Heger ('04) introduced metallic powder into the peritoneal cavity and studied its movement by means of the x-ray. He believed that granules of particulate matter entered the lymph vessels of the omentum and were carried in the lymph stream to the left lobe of the liver. Norris ('08) and Koch ('11) described numerous lymphatics in the dog omentum. By means of silver nitrate preparations, Casparis ('18) demonstrated omental lymphatics along the large blood vessels, but he failed in all attempts to inject these channels with color masses. In the same year Baum ('18) summarized the results of his

extensive studies of the lymphatic system in the dog. He described omental lymphatics which accompany the blood vessels in the fat strips. By injecting these channels with color masses he demonstrated that the lymph vessels of the omentum drain into nodes near the duodenum and spleen, and from these collecting points the drainage is successively to the portal nodes, the intestinal lymph trunk, and the cisterni chyli.

Recent investigators are apparently unaware of Baum's observations. Hamazaki ('25) noted numerous lymph channels in the milk spots of the dog omentum but did not discuss the connections between these vessels and those described by previous workers. In his omental studies of various laboratory animals, presumably including the dog, Poynter ('28) does not mention the presence of lymph channels. Higgins and Bain ('30) studied sections of dog omenta but do not record that lymph vessels were observed. This omission indicates that many present day anatomists doubt the existence of omental lymphatics in the dog.

The presence of lymph channels in the cat omentum has been generally denied since the publication of Ranvier's observations (1896). He reported that such channels occur only in the newborn kitten and they disappear by regression, leaving no indication of their existence in the adult. Shipley and Cunningham ('17) were unable to identify lymph channels in the sections of cat omenta which they studied, and Hamazaki ('25) stated that the lymph vessels in the milk spots are not clearly seen. Poynter ('28) studied sections of omenta from various laboratory animals but did not report the occurrence of omental lymphatics in the cat. In their recent study of this animal, Higgins and Bain ('30) isolated the distal portion of the omentum in a subcutaneous pouch. A few days later they injected a graphite suspension into the pouch, after which animals were killed at successive intervals and regions of graphite infiltration noted. On the basis of these observations they state that the routes of drainage from the distal part of the omentum involve the gastrolienal ligament, the

dorsal surface of the stomach, the lesser omentum and caudate lobe of the liver, the coronary ligament of the liver, the lymph vessels of the central tendon of the diaphragm, and the anterior mediastinal lymph nodes. These investigators were unable to demonstrate conclusively the presence of omental lymphatics in this animal. It appears, therefore, that anatomists in general agree with Ranvier's statement that omental lymph vessels do not occur in the adult cat.

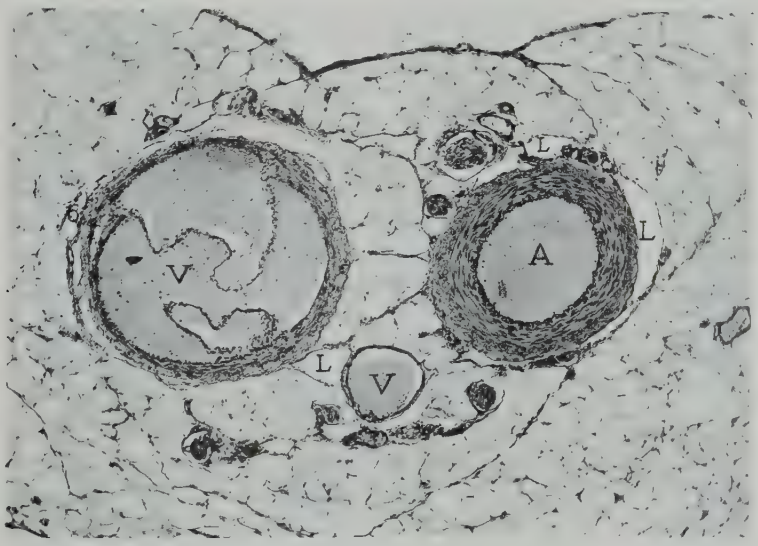


Fig. 1 Photomicrograph of a section of dog omentum showing an artery, A; veins, V; the larger with valve, and numerous lymphatic channels, L. Three nerves are shown to the left of the arterial wall. × 60.

OBSERVATIONS ON THE DOG OMENTUM

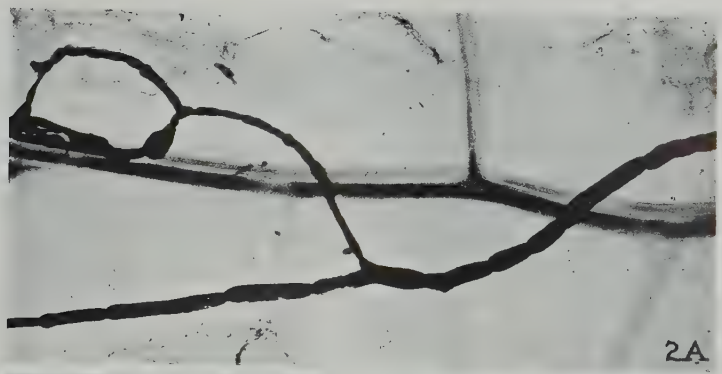
In view of the lack of agreement concerning the existence of lymphatics in the omentum of the dog, serial sections from different parts of this organ were prepared and studied. In every preparation numerous lymph channels were easily identified in the fat strips which accompany the blood vessels (fig. 1). These lymphatics consist of an anastomosing plexus of thin walled vessels surrounding the artery, and one or more

solitary channels, located in the perivascular fat some distance from the blood vessels. The channels comprising the arterial plexus lie adjacent to the adventitia of the arterial wall and are often much compressed. The outlying or solitary vessels are usually larger in size and appear almost circular in transverse section. All omental lymphatics are lined with a distinct endothelium and have numerous valves.

A more complete picture of the omental lymph vessels in the dog was obtained by injecting them with filtered India ink. This was accomplished by forcing the point of a small (27 gauge) hypodermic needle through the perivascular fat until it was quite close to the arterial wall and almost parallel with it. A slight amount of pressure was then applied to the plunger of the syringe. In this manner the arterial lymphatic plexus was often punctured and filled with the injection fluid. It was observed that the ink never extended in the plexus for a distance of more than 1 or 2 cm.; instead, it passed through connecting branches to the solitary channels (fig. 2 A). The number and position of these channels are variable. A single one, lying near one margin of the perivascular fat is a frequent finding, but it may cross the blood vessels from one side to the other and be accompanied by one or more lymph vessels of the same type (fig. 2 B). These continue as tortuous channels through the perivascular fat to the proximal attachments of the omentum. Along the course of such channels numerous ink-filled spurs of tributaries may be observed (fig. 2 C). Further extension of the injection fluid into these tributaries was barred by their most proximal valves.

It was not possible to fill a large number of lymph channels in any one omentum, for the ink smudge resulting from

Fig. 2 Photomicrographs of portions of dog omenta in which the lymph channels are injected with India ink. × 8. A, a short plexus of lymph vessels surrounds the artery and empties by communicating branches into a more outlying channel. Note the beaded appearance of the latter, indicating the position of valves. B, several lymph channels accompanying the blood vessels. C, a dense lymphatic plexus obscures the blood vessels. Outlying channels, one with a spur, S, also shown.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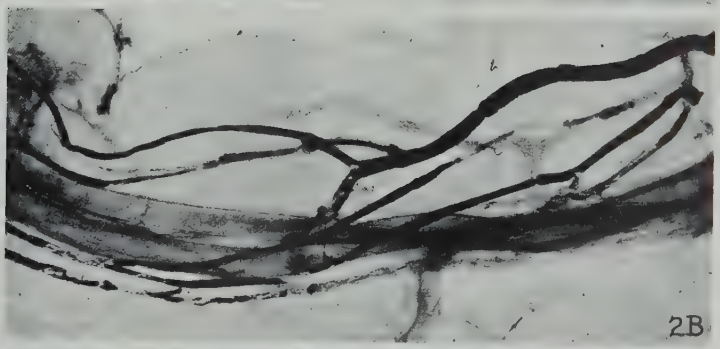
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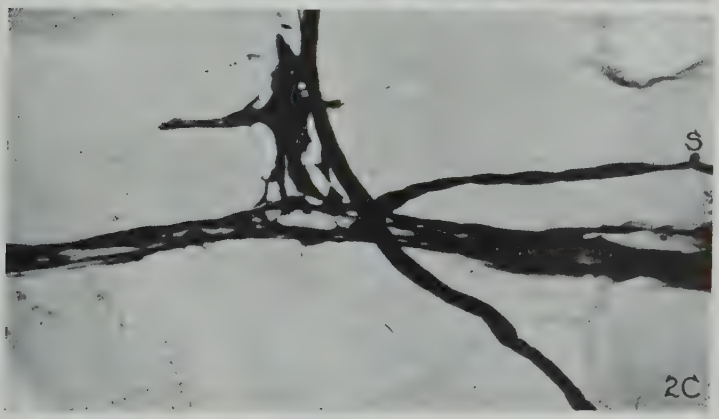
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2C

numerous attempts soon clouded the entire organ and made further efforts impractical. Best results were obtained on fresh material[1], for in such omenta the perivascular fat is relatively transparent and the ink-filled channels can easily be identified and followed. By means of repeated injections in all regions, the drainage paths of the lymphatics in the dog omentum were established (fig. 3). Almost all of the lymph vessels of the ventral leaf drain into transverse connecting channels which lie near the gastroepiploic blood vessels. These lymphatics empty their contents into the duodenal lymph node. The lymph from a relatively small area at the left side of the ventral leaf drains into collecting channels which accompany the left gastroepiploic vein to lymph nodes near the spleen. The splenic nodes also drain the left one-third of the dorsal leaf of the omentum, while lymph from the remainder of this leaf enters the duodenal lymph node directly or through efferent channels from a minute omental node which lies 2 or 3 cm. distal to the duodenal node and is frequently absent. It may be noted that these observations are not entirely in accord with the statements by Baum ('18) that the duodenal node drains the right half and the splenic nodes the left half of the ventral leaf of the omentum.

The further course of the lymph stream from the duodenal and splenic nodes was also determined by injection. Efferent channels from the duodenal nodes empty into nodes located on either side of the portal vein. Ink injected into these portal nodes drained into the intestinal lymphatic trunk, and then into the cisterni chyli. Efferent vessels from the splenic nodes pass either to the portal nodes or directly to the intestinal trunk. There was no indication in any of these experiments that the paths of omental drainage in the dog involve the lymph vessels of the diaphragm.

[1] The writer wishes to thank Doctor Higgins and Mr. Helfrich, of the Pharmacology Department, University of Illinois, College of Medicine, for their cooperation in securing material for this investigation.

OBSERVATIONS ON THE CAT OMENTUM

When attention was directed to serial sections of omenta from adult cats, it was noted that lymph channels are a constant finding. This observation deserves emphasis in view of the statements by Ranvier (1896) and Shipley and Cunning-

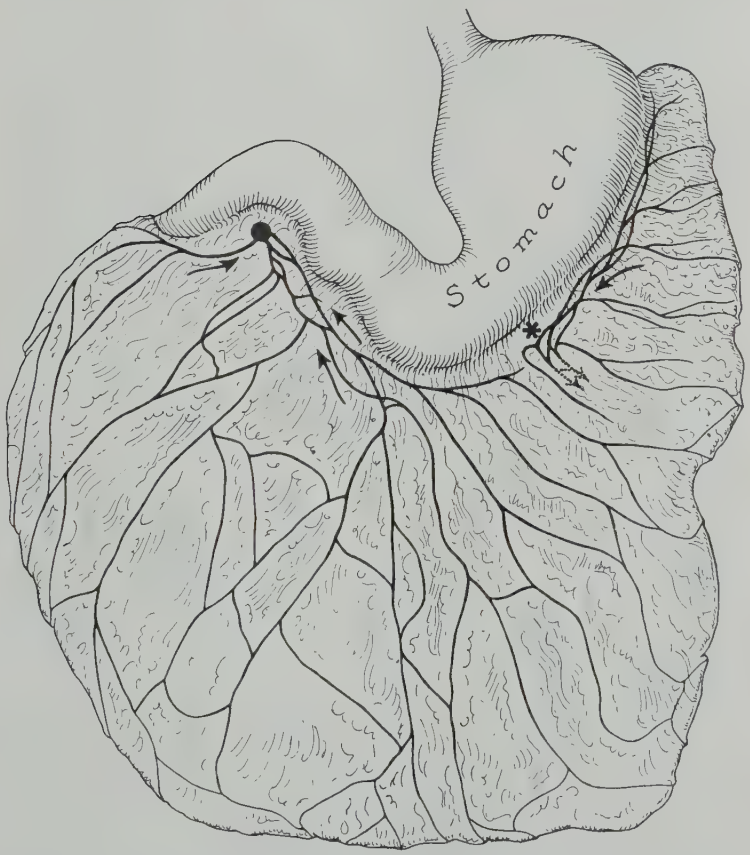
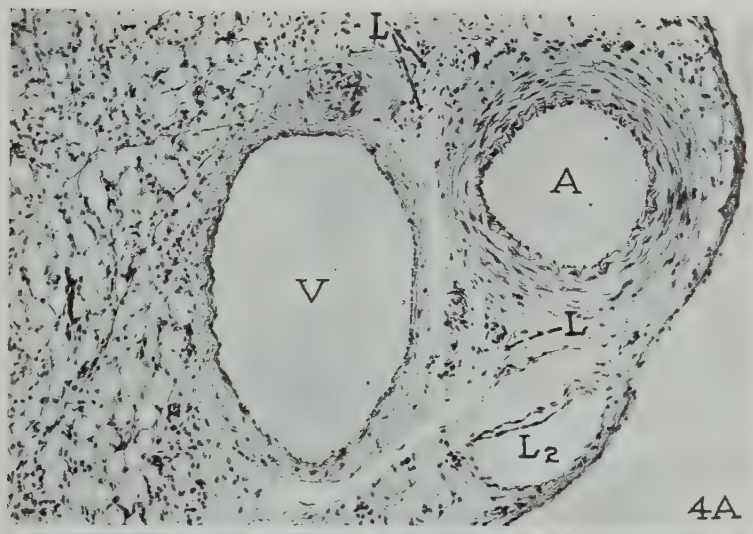


Fig. 3 Drawing showing the drainage paths of the lymphatics in the ventral leaf of the dog omentum. The black spot near the duodenal flexure is the duodenal lymph node. The asterisk indicates the point at which the lymph vessels draining the left portion of this omental leaf pass dorsally, in company with the gastroepiploic vein, to empty into the splenic lymph nodes. Four-ninths natural size.

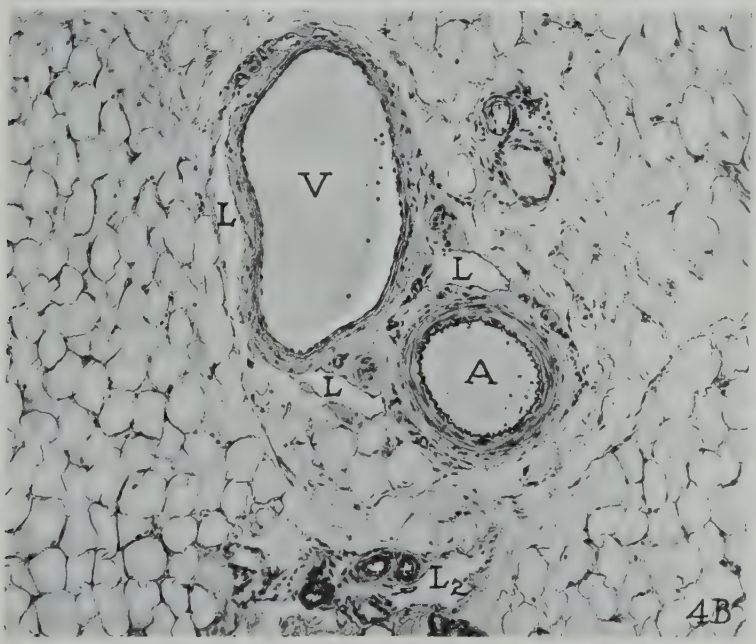
ham ('17) that such vessels are not present in the omentum of the adult cat. But the existence of omental lymphatics in this animal cannot be doubted, for they may be observed in preparations from all parts of this organ. The omental blood vessels in the cat are much smaller than in the dog, and as a consequence the lymph vessels which accompany them are neither as large nor as thick walled as those described in the larger animal, but they have a distinct endothelial lining and numerous valves. Central lymph channels are located between the blood vessels in the perivascular tissue (fig. 4), but rarely form a plexus around the artery. Occasionally an outlying channel is observed near the blood vessels (fig. 4 A). Ink injections accomplished with difficulty in this animal, show that the central channels pass in a relatively straight course between the blood vessels (fig. 5, A, B and C). The absence of numerous anastomosis between these channels is a characteristic feature in the cat. Communicating branches connect such central lymphatics with the small solitary channels which carry the lymph to the collecting nodes, but which are not numerous in any specific locality and do not show as frequent branching as was observed in the dog, though they have the typical segmented form (fig. 5). In addition to the normal inflation of the proximal portions of these vessels, it is not unusual to find them filled with ink for a short distance distal to the point of injection. This indicates that even the slight back pressure which is employed is sufficient to overcome the action of the nearest valves.

The paths of lymph drainage in the cat are similar to those in the dog, although the number and position of the lymph nodes often show considerable variation. A single node is located along the median side of the duodenum at the level of its superior flexure, and ventral to the pancreas. In one of the eighteen animals examined a second node was observed

Fig. 4 Photomicrographs of sections of cat omentum. × 120. A, vein, V; an artery, A; central lymph channels, L; and solitary lymph channel with valve, L_2. A large nerve lies above the vein. B, central lymph channels, L, between the artery and vein, and a communicating lymphatic, L_2.



4A

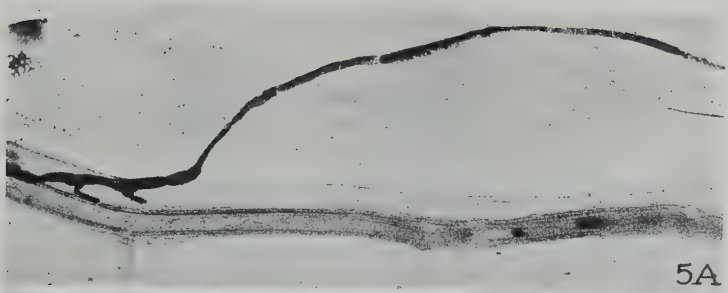


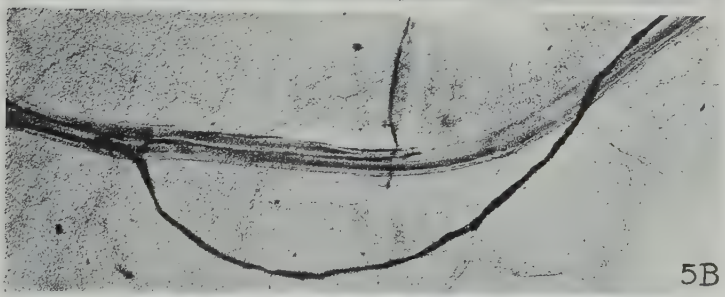
4B

at the same level, but on the lateral side of the duodenum. The splenic nodes consist of one or more small glands located at the distal border of the hilus of the spleen, and a single much larger gland which lies near the point of union of the gastric and splenic veins. The duodenal and splenic nodes are the primary collecting centers of the lymph stream from the omentum, and their efferent channels lead directly into the intestinal lymph trunk or into the nodes located near the portal vein. A left portal node was present in every animal examined, and in two cases a smaller right portal node was observed. When India ink is injected into the portal nodes, it passes rapidly into the intestinal lymph trunk and cisterni chyli and then fills the entire thoracic duct. There was no evidence in these experiments that the lymph from omental vessels drains into the thoracic duct by paths other than those just outlined. Higgins and Bain ('30) described transference of particulate matter from the greater omentum of cats by paths which follow the dorsal surface of the stomach, the lesser omentum and the diaphragm. It must be emphasized that that they did not observe definite lymph channels in the greater and lesser omenta in which the particulate matter was being transferred, and it is therefore apparent that the paths described by them are not those concerned with omental lymph drainage.

In all sections examined in this study, omental nerves were a constant finding. The existence of such nerves has been doubted by many investigators, but there is no question that they occur generally for they have been observed recently also in the human omentum by the writer ('34).

Fig. 5 Photomicrographs of portions of cat omenta in which the lymph vessels are injected with India ink. × 16. A, B, and C, central lymph channels accompany the blood vessels and drain into outlying collecting channels. An ink-filled valve sinus is seen near the bifurcation of the artery in C. D, an outlying lymph channel. The sharp angle near its center indicates the presence of a valve at the junction of a tributary.


5A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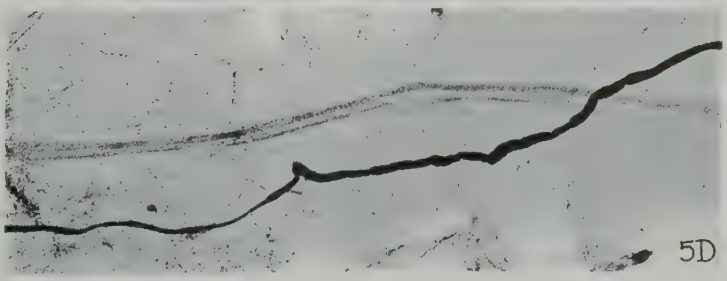
5D

SUMMARY

1. Omental lymphatic channels accompany the blood vessels in all parts of this organ in the adult dog and cat. These lymphatics consist of central channels which lie near the blood vessels and communicate at intervals with more solitary channels which lie peripherally in the perivascular fat.

2. Omental lymph drainage is by way of such outlying channels into the duodenal and splenic lymph glands. Lymph from these nodes passes directly or indirectly into the intestinal lymph trunk and cisterni chyli.

3. There is no evidence that the lymphatic channels of the diaphragm play any part in omental lymph drainage in the dog and cat.

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THE CELLULAR CYCLE, THE GOLGI APPARATUS AND THE PHENOMENON OF REVERSAL IN THE HUMAN THYROID PARENCHYMA [1, 2]

J. GILLMAN

Department of Anatomy, University of the Witwatersrand, Johannesburg

FOUR FIGURES

INTRODUCTION

Since 1898, when Golgi first recognized its constant presence in the nerve cell, the majority of investigations upon the Golgi apparatus have been directed toward proving or disproving its reality. Even today there are those who admit that the Golgi apparatus can be seen in cells suitably treated, but dispute its integrity as a specific cellular constituent, and doubt its significance because of its fluctuant and variable character. Bowen ('27), on the other hand, realized that this fluctuant and variable character of the Golgi apparatus is its most important attribute. He recognized that its morphology varies according to the physiological state of the cell. Next to the mitochondria, it is the most valuable and sensitive indicator we know of cellular activity.

Most of the investigations on the Golgi apparatus have been performed on exocrine glands, such as the submaxillary gland, pancreas, liver, intestine and breast. The Golgi apparatus undergoes definite changes during physiological activity and yet throughout these changes it maintains a relatively stable position in the cell. Lying on the luminal side of the nucleus and consequently in that half of the cell

[1] I have not attempted to discuss the question of the 'parafollicular' cells or other parenchymal elements in the thyroid gland, since it would take us beyond the confines of the present paper. Only those cells which take part in the formation of follicles have been considered.

[2] Received for publication, March, 1934.

from which secretion is pouring, it serves as an indicator of cellular polarity.

In endocrine glands where there is no duct to inform us precisely in what direction the secretion is passing, this 'luminal' constancy in position of the apparatus, established for exocrine glands, can be utilized to elucidate the direction in which the secretion is flowing. The thyroid gland in particular, on account of its follicular or luminal arrangement, has been regarded as a stepping stone to the understanding of secretory polarity in other endocrine glands that have not even a lumen.

Cowdry ('21) proved that in the thyroid gland the follicle in which colloid is stored represented the remains of the ancestral lumen of the gland. The primary direction of secretion is toward the follicular lumen and not toward the blood vessels as Norris first suggested. Cowdry adduced confirmatory evidence of this fact by the distribution of the Golgi apparatus, which he found to be situated between the nucleus and the follicular lumen. This seemed to indicate that the thyroid, like other exocrine glands, had definite luminal secretory polarity.

Later, on the basis of observations which seemed to him to prove that the Golgi apparatus in the adult guinea pig thyroid gland migrated from one part of the cell to the other, Cowdry ('22) said that, "Apparently in the thyroid gland alone is there variability in position of the reticular material, and in the thyroid alone have we clear evidence of physiological reversal in the direction of secretion." He therefore believed that an endocrine gland such as the thyroid could pour its secretion either in the direction of the lumen or of the peripheral blood stream. This statement was the starting point of the present investigation. In attempting to confirm or disprove his thesis, I have found it necessary to examine in more detail other associated cytological features of thyroid structure, such as cell volume, cell shape, nuclear and other cytoplasmic inclusions.

In this paper it is my main purpose to show that the shape, size, form and distribution of the Golgi apparatus are reliable diagnostic criteria of secretory activity in the thyroid gland and that, utilizing these criteria, we can establish a rational nomenclature. I hope also to demonstrate that a nomenclature of this sort is very necessary in order that the Golgi apparatus of cells in similar physiological states may be compared, and that the lack of it has been responsible for fallacious interpretations that have arisen concerning the size and position of the Golgi apparatus and concerning the mechanism whereby colloid is absorbed into the blood stream.

MATERIAL AND METHOD

Human, baboon, guinea pig, dog and mouse thyroid glands were used for this investigation. The human material consisted of four normal thyroids obtained at execution and removed from Bantu males of ages varying between 26 and 54 years, and of three abnormal thyroids removed at operation under nitrous oxide anaesthesia from Europeans. Two of these thyroids—one from a male, the other from a female—were from patients exhibiting all the symptoms and signs of thyrotoxicosis. The third abnormal thyroid was removed from a European female, aged 49 years, who had complained merely of an ungainly swelling in the neck.

The baboon, guinea pig, dog and mouse material was utilized to confirm the general applicability of our thesis and has not been used in illustrating the paper. The pathologically hyperactive thyroids were employed in order to determine whether the direction in which the secretion passes in such thyroids, as ascertained by the position of the Golgi apparatus, is different from that in the normal thyroid gland. It was found that the essential cytological difference between the normal and abnormal hyperactive thyroids is of degree rather than of kind. On the basis of this fact, the illustrations embodied in this paper were made from the hyperactive thyroids because the various stages of secretory activity are more obtrusively demonstrated there.

The human thyroid tissue was fixed within 5 to 10 minutes after removal at operation or at execution, and the remaining animal tissue while the heart was still beating. The animals were killed by striking them with a heavy object over the atlanto-occipital joint. Three methods were used to demonstrate the Golgi apparatus, namely, the Mann-Kopsch method, Cajal's uranium nitrate method and Da Fano's modification of the Cajal method.

The Mann-Kopsch method, when successful, yields the most brilliant pictures, but its capriciousness and the time consumed during impregnation render it, in our experience, unfavorable for routine examination of the Golgi apparatus in the thyroid gland. The method of Da Fano ('22), on the other hand, has the excellence of consistency. With his technique we have rarely failed to demonstrate the Golgi apparatus in the thyroid gland, and some of the ordinary preparations compare favorably with the best of Mann-Kopsch series. Da Fano's method has much to recommend it, because mitochondria, secretory granules and other bodies of a lipoidal nature are also impregnated. Throughout this investigation the material relied upon was that prepared by this method, although frequent reference was naturally made to the Mann-Kopsch series.

The investigation has also shown that to avoid fallacy in studying the Golgi apparatus sections must be mounted serially. Further, in order to have a three-dimensional picture of the apparatus, Professor Dart suggested the reconstruction of single cells in celluloid. By this method the exact localization and the ramification of the bulk of the apparatus in the thyroid cell were depicted. It was rather difficult to reconstruct the finer arborizations of the Golgi apparatus. At the same time the gross relationship of the apparatus to the nucleus has been determined. By this method, too, it was possible to determine approximately the fluctuating volume of the thyroid cell during its various phases of physiological activity.

The cell selected for reconstruction, under 1350 times magnification, is accurately measured by means of an oculomicrometer and the parts required are plotted on graph paper at a still larger magnification. By this means a tolerably precise outline of the cell, Golgi apparatus and the nucleus is obtained. For increased accuracy measurements and drawings of this sort were made at high, middle and low focus on each section. A strip of celluloid is then placed over each drawing and the outline of the structures is scratched on the celluloid with a sharp needle. The outline of the cell wall is cut out with a pair of scissors and the magnified nucleus and Golgi apparatus then painted with different water colors. The plates, built up in order, are spaced at correct distances by the introduction of small extra pieces of celluloid between them in the customary manner, and are made to adhere to one another by means of a few drops of acetone. The advantages of this method are that it is cheap and quick, and by its means the cytological arrangements may be viewed transparently as it were, when the reconstruction is held up to the light.

ACKNOWLEDGMENTS

I am deeply indebted to Prof. R. A. Dart for his invaluable advice and encouragement in this work.

Dr. A. Galloway gave me useful assistance at the inception of the work. Mr. H. B. Stein has assisted me particularly on the technical side and, through his own research, was responsible for collecting the pathological material that formed an important nucleus for the paper. Mr. Schepers has been responsible for the illustrations, and to Doctor Haines of the University of Cape Town I am indebted for the technique of celluloid reconstruction.

THE GOLGI APPARATUS IN THE THYROID DURING SECRETION

Before naming and describing the variations in position of the Golgi apparatus in the thyroid cell, it will be advantageous to review the present extent of our knowledge con-

cerning these variations. It is a common enough phenomenon when examining single sections of the thyroid gland to discover a Golgi apparatus that lies between the nucleus and the base of the cell. The first person to draw particular attention to, and put forward a reason for such a basal or 'reversed' position of the apparatus was Cowdry. He suggested that, in the adult guinea pig thyroid gland, this position is achieved by a migration of the apparatus around the nucleus from the luminal to the basal pole. Cramer and Ludford ('26) studied the Golgi apparatus in different stages of secretory activity induced experimentally. In so-called 'resting cells' they described a Golgi apparatus closely applied to the nucleus but on its external or luminal aspect. When the functional activity of the cell was increased the apparatus underwent great enlargement extending from the nucleus in the direction of the lumen of the follicle. They found no evidence to support Cowdry's hypothesis that the Golgi apparatus migrated from the apex to the base of the cell when subjected to phenomenal changes in functional activity. When the Golgi apparatus was noticed by Ludford and Cramer to lie occasionally on the basal side of the nucleus and thus away from the follicle, they attributed it to 'mechanical displacement.'

Shiro Ishimaru ('26), after extirpation of three-quarters of the thyroid gland in the rabbit, believed that definite changes of a migratory character did occur in the Golgi apparatus. About 6 to 12 days after the operation the apparatus enlarges and the basal position is frequently seen. He, therefore, agreed with Cowdry that the Golgi apparatus does migrate from one pole of the cell to the other. Later Ludford and Cramer ('28) in describing the cytological changes in the thyroid gland of two cases of exophthalmic goitre—one in man, the other in the mouse—recognized great enlargement of the Golgi apparatus, which extended widely into all parts of the cytoplasm. In many of these exophthalmic goitrous cells they described the apparatus as being situated between the nucleus and the base of the cell and agreed with Cowdry's

hypothesis. The enlargement of the Golgi apparatus they described in exophthalmic goitre was confirmed by Okkels ('31) and is an unqestionable fact.

Ingram ('30) demonstrated in Necturus that in the resting cell the Golgi apparatus formed a perinuclear cap, situated toward the apical or luminal pole of the cell. During activity the Golgi apparatus increased in size but was always located on the luminal side.

From the above investigations it can be generally recognized that the Golgi apparatus varies in size and appearance during cellular metabolism, but doubt exists as to whether it migrates and as to the position it assumes in the cell. Both of these aspects as observed in the human thyroid gland will now be considered.

OBSERVATIONS

As we have seen, the thyroid cells vary in structure according to their functional state; but as yet, no attempt has been made to define these states by describing the anatomical arrangements that characterize them. If such anatomical variations occur regularly, it is eminently desirable that they be classified so that each observer may understand more precisely the observations of his fellow. I have, therefore, divided the physiological cycle for descriptive purposes into three stages, which, although not so named, can be regarded as implicit in the literature, i.e., the resting stage, the anabolic stage during which the secretion is being elaborated, and the katabolic stage or stage of liberation of secretion from the cell. It will, of course, be realized that, as in the phases of mitosis, each separate stage merges insensitively into the succeeding one.

There is, in addition, a fourth stage about which little seems to be known. It is sometimes found in normal resting thyroids and sometimes in abnormal glands. It is seen to advantage as a normal condition in the thyroid of the hibernating frog (Ingram, '30), and the same type of cell is found in the abnormal condition in the colloid goitre of man. H. B.

Stein has produced experimentally this type of cell in guinea pigs during chronic adrenaline intoxication. This type of cell is neither resting, anabolic nor katabolic. It is inhibited. But as the consideration of this cell would take us beyond the confines of our present paper, it is mentioned merely for completeness and will not be discussed further.

In order to name cells satisfactorily in this fashion, criteria must be laid down and such criteria are readily accessible for they are those which evidence anabolism or katabolism, viz.: shape and size of the cell itself, its nucleus, its Golgi apparatus, as well as the presence or absence of secretory granules, and the nature and distribution of the mitochondria.

Figures 1 a–c represent reconstructions of cells at a magnification of 1000 times. Figure 1 a shows the anabolic cell, figure 1 c the katabolic cell, and figure 1 b the resting cell. With regard to these reconstructions, it is to be noted that the thyroid cell in horizontal section usually has a hexagonal cell boundary. In making the reconstructions it was not found possible to reproduce accurately this form of the walls, and the cells have, therefore, been reconstructed as though they were rectangular prisms. It must be recognized that the reconstructions are only approximately correct, but what they lose in width they gain in all probability in length. In any case, since the method of reconstruction has been the same in each case, each of the cells is strictly comparable in form and volume with its fellow.

The anabolic cell in man (fig. 1 a and fig. 2) according to preparations by Da Fano's method, is polygonal in shape and is limited by a regular cell membrane which bulges with a regularly-curved contour in the direction of the lumen of the follicle. In early anabolism the volume of the cell is approximately 10,000 cu. μ. The average height of this cell taken over 20 typical cells is 27 μ, the width 18 μ and the length 20 μ. The nucleus of which only the nuclear membrane is defined, is either oval or spherical, and is located toward the centre of the cell. The cytoplasm is filled throughout with numerous faintly-impregnated argentophilic granules, which are either

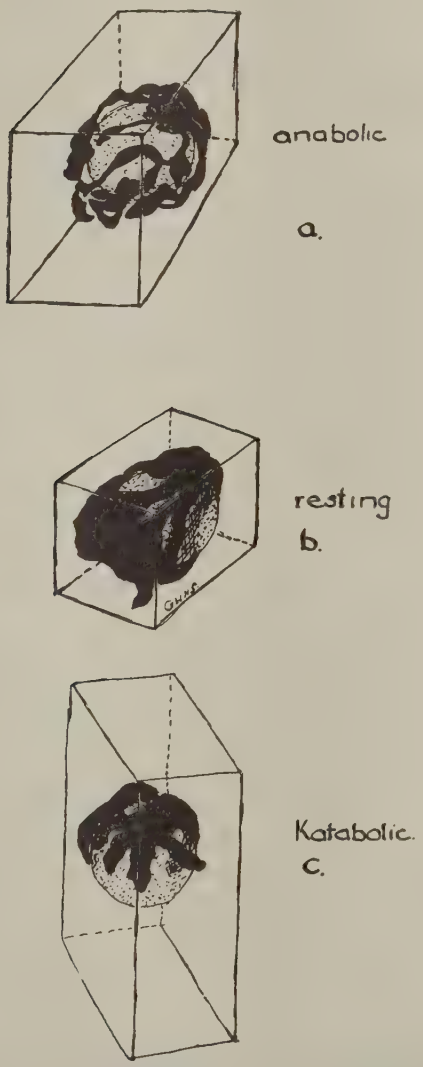

Fig. 1 Drawings of reconstructions of the thyroid cell during physiological activity to exhibit its general morphology and the variations in the Golgi apparatus. × 1000.

secretory granules or mitochondria or both. The Golgi apparatus is a large, loosely-woven network very intimately related to the nucleus around which, in the majority of cases, it forms an almost complete openwork cap (fig. 2 b). The meshes of this network are wide, and often the loops extend for some distance into the adjacent cytoplasm. Varicosities may be observed at intervals along the filaments of the apparatus. The illusion of swellings is due chiefly to folds and bends in the structure as it dips into the deeper portions of the section. I have found no evidence, as Shiro-Ishimaru suggested, that the peripheral filaments were connected with the terminal bars, or that they were in any way attached to the cell membrane. This anabolic cell, which is in a state of great engorgement and intracellular tension, then passes into the second or katabolic stage.

The appearance of the cell during the katabolic stage can be better appreciated if this stage is regarded as having two phases; namely, the early and the late katabolic phases. The early katabolic phase (fig. 2 c) presents a vivid contrast with the anabolic stage.

In vertical section the cell has no longer a polygonal outline, but is tall and columnar and bulges irregularly into the follicular lumen. Taking an average again of over twenty typical cells, they measure approximately 40 μ in height, 15 μ in width and 18 μ in length and have a volume of approximately 11,000 cu. μ. The increase in cellular height with decrease in the width is responsible for the papillary ingrowths that occur in hyperactive follicles, both normal and abnormal. The early katabolic nucleus is situated toward the centre of the cell having migrated away from the base. I can confirm Ludford and Cramer's observation that the nuclear membrane is more heavily impregnated in the secretory or katabolic cell. The cytoplasm is also distinct from that of the anabolic cell, for it is filled with very coarse granules. These are restricted to the apical portion of the cell and are evidently secretory in nature; but finer granules are also present chiefly toward the base of the cell. The coarse granules vary in size, but

on an average they are about the size of streptococci. These dark brown granules have in their midst fewer but yet numerous black granules about twice the size of the secretory granules. The black granules are of an unknown lipoidal nature. The Golgi apparatus in this phase is contracted and not branched. It is now a dark massive structure located upon the luminal or apical surface of the nucleus (figs. 1 c, 2 c–d). Very little of its detailed structure can be observed in the katabolic cell owing to its strong and characteristic affinity for silver. It still hugs the nucleus very closely.

As the elaborated secretion is discharged from the cell, the intracellular pressure must diminish. This produces in turn an irregular and collapsed contour of the free portion of the cell which now enters into the second phase of the katabolic condition (fig. 2 d). The nucleus is centrally placed and has a deeply-impregnated nuclear membrane which becomes regressively less heavily impregnated as the resting stage is approached. The larger granules disappear, but numerous faint, dust-like argentophilic granules are now seen toward the apex enclosing clear areas in the cytoplasm. These clear areas are vacuoles and probably correspond with those described by Ludford and Cramer in Mann-Kopsch preparations. These vacuoles are in no way related to the Golgi apparatus, but may be the areas previously occupied by the apparently solid secretory granules of the early katabolic phase. The Golgi apparatus is now much reduced in size and forms a closed dense reticulum.

The irregular contour of the cell membrane, the loss of impregnated substance in the cytoplasm, the presence of vacuoles and the dense Golgi apparatus only capping the nucleus are characteristic features of the late katabolic stage.

I discovered no evidence that any fragmentation of the Golgi apparatus occurred or that fragments reunited to form a dense network at any phase during katabolism, neither was there visible any extrusion of a portion of the Golgi apparatus with the cellular secretions.

The third phase is that of rest (figs. 1 b, 2 a). It is this stage which is generally described in textbooks of histology as being typical for the normal thyroid cell. In this stage the process of active secretion is at an end. The cell after losing its polygonal outline becomes cuboidal having for twenty typical cells the following average dimensions: 14 μ in height, 15 μ in width and 18 μ in length with a volume of approximately 3000 cu. μ. In other words, the resting cell is roughly one-third the size of the early katabolic cell and one-quarter the size of the anabolic cell. The centrally-placed nucleus limited by the well-defined nuclear membrane is oval. The cytoplasm exhibits numerous punctate argentophilic granules which are smaller and more discrete than in the anabolic cell. The Golgi apparatus becomes even more concentrated than in the katabolic cell into a simple coarse reticulum capping the apical aspect of the nucleus and extending for some distance over the lateral extremities of the oval nucleus. As reconstruction of this cell shows, the Golgi apparatus is not nearly so slender as its rod-like appearance in sections suggests and as it is generally depicted (figs. 2 a, 1 b). It is a relatively massive structure for so small a cell. In sections which include only the lateral portion of the nucleus the Golgi apparatus may appear to have a complete perinuclear relationship and its massiveness is readily appreciated.

Although it is difficult to obtain even approximate figures for the volume of the Golgi apparatus, it is legitimate to infer from the reconstructions that the volume does not vary very much during the secretory phases. The changes that take place in the apparatus are rather such as would increase its surface area in the anabolic cell by an unraveling of the dense reticulum seen in the resting stage and so producing the rich arborescence characteristic of the anabolic stage.

From the above the chief changes that occur in the thyroid cell during secretory activity can be summarized as follows (fig. 2 a–d): The cell volume more than trebles its capacity from the resting stage to that of beginning to pour out its secretion. The cytoplasmic constituents such as mitochondria,

Golgi apparatus and secretory granules show distinct changes during these stages in secretion of the cell. From the consistent relationships between the Golgi apparatus, mito-

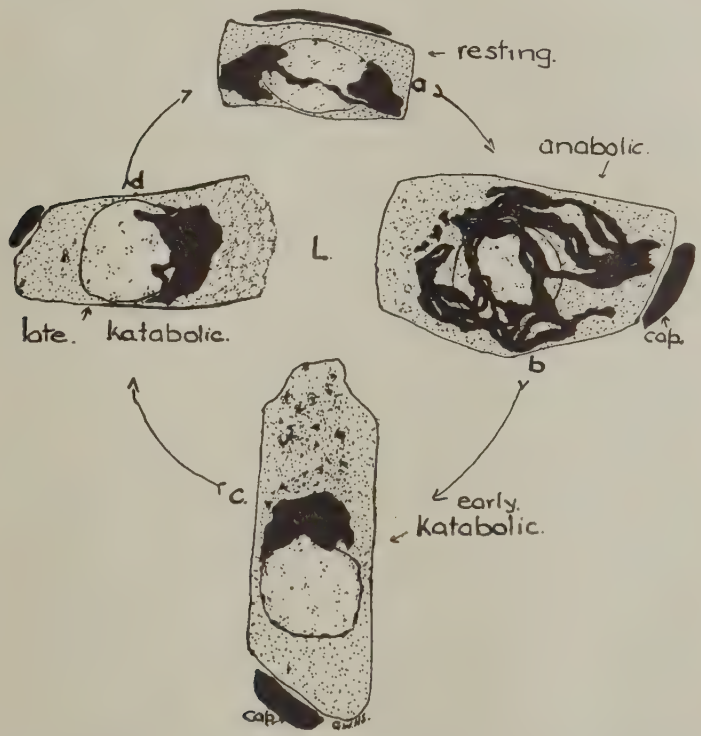

Fig. 2 Pictographic study from sections of human thyroid cells during secretion. L = position of the lumen of the follicle. Cap = capillary at the base of the cell. 5/4 x 1000.

chondria and secretory granules, and the shape and size of the cell, it is possible to diagnose the physiological state of any thyroid cell by a consideration of any one of the above anatomical features.

CHANGES IN THE GOLGI APPARATUS IN THE THYROID GLAND AND IN OTHER GLANDULAR EPITHELIA DURING THE PHASES OF PHYSIOLOGICAL ACTIVITY

The changes which occur in the configuration of the Golgi apparatus in the thyroid cell are not isolated phenomena. Similar fluctuations characterize the apparatus in all body cell, and it is serviceable to show here that from the earliest work onward investigators have recognized these fluctuations. Golgi (1899), the original discoverer of the apparatus, was occupied not only in proving its existence, but in observing its changes. Having examined the intestinal epithelium of various vertebrates, especially that of the frog's stomach, he showed that in cells where the amount of mucin is small the Golgi apparatus is diminutive and situated near the luminal pole of the nucleus. Cells in which a great deal of mucin is accumulated, on the one hand, have a large, robust reticular apparatus which is displaced toward the base of the cell.

Cajal ('10), following up Golgi's work in proving the reality of the Golgi apparatus, extended his observations on the intestine. He concentrated on these changes in the apparatus and showed that, even after the goblet cells have discharged their elaborated secretions, the Golgi apparatus is still present, but only in the form of one or two clumps located on the luminal side of the nucleus. When further mucinogen is elaborated in the goblet cells, these cells increase considerably in size and at the same time the reticular apparatus becomes an enlarged and complicated network. Just as the cell is about to extrude its secretion, the network fragments into dust-like particles which are thrown off into the lumen together with the secretion. From these observations, then, we are in a position to state that the Golgi apparatus in the intestinal mucosa fluctuates in size and form from almost invisible scattered dust through threads to a complex easily visible network that once more disintegrates into dust.

Cajal ('14) confirmed the soundness of his earlier observations on the changes found in the apparatus in the intestinal mucosa by investigating those taking place when the sub-

maxillary glands and the pancreas are experimentally treated with drugs. In these investigations, while transformation of the reticular apparatus into secretory products could not be demonstrated, it seemed clear that, at the height of functional activity in the submaxillary and pancreatic cells, there is an accompanying enlargement of the apparatus. Da Fano ('22) also displayed changes in the Golgi apparatus, selecting for his purpose the mammary glandular epithelium in its markedly different physiological states. He found that the small reticular apparatus of the virgin breast hypertrophies in all directions in the cytoplasm during pregnancy (anabolism) and tends to surround the nucleus. Before suckling has occurred, when the secretion has been poured out, and as yet no stimulus has occurred for further secretion, the apparatus decreases in size and assumes a 'roundish or oval shape.'

But the most exhaustive account of the changes in the Golgi apparatus during secretion is that given by Nassonov ('23). Nassonov, in showing the relationship of the Golgi apparatus to the secretory granules, demonstrated incidentally that the columnar or pre-secretory cells have a Golgi apparatus consisting of a delicate network. When the secretory droplets increase in size, the reticular apparatus increases pari passu until the height of activity is reached. The network at this stage caps the nucleus, overlapping it to a great extent along its circumference. As secretion diminishes the Golgi apparatus contracts from the basal end of the cell. It does not break up or mingle with the secretion. Bowen ('24) repeated Nassonov's work and confirmed his findings. Ludford and Cramer ('27) described the variations of the Golgi apparatus in the islets of Langerhans of the pancreas during the metabolism of those cells. As their pictographic study shows, there is a great enlargement during activity and a reduction in size during inactivity. They also found fluctuations in the thyroid Golgi apparatus during their researches on its physiological states.

From my own observations it is apparent that the Golgi network varies in an orderly and regular fashion. It unravels

itself from the dense rod-like apparatus of the resting cell to the richly arborizing apparatus of the anabolic cell. During katabolism there is again a concentration of the apparatus. Throughout all these mutations the Golgi apparatus preserves its nuclear relationship which is certainly very intimate.

It is obvious, therefore, that the metamorphoses of this apparatus occur during physiological activity throughout the various organs and tissues of the body. It is for this reason and because all the body cells are subject to the same rhythms of anabolism and katabolism that I have based my terminology of thyroid cytology on a physiological concept.

'REVERSAL' OF THE GOLGI APPARATUS IN THE HUMAN THYROID GLAND

The direction in which the thyroid secretion passes has been a very difficult problem to settle. When the colloid in the lumen is considered by some to be a reserve secretion over and above the amount required for the time being by the body, others maintain that the colloid is a waste product of secretion.

I have already referred to the reversal reported by Cowdry ('22) and the argument he based upon that reversal concerning the direction of secretion in the thyroid gland. Ludford and Cramer ('28), after preliminary failures to recognize reversal, were ultimately led to extend Cowdry's hypothesis to the human thyroid gland. They thought that, whereas the reversed position never occurred in the normal human thyroid glands, it could frequently be detected in pathological hyperactive conditions, e.g., exophthalmic goitre. From this Ludford and Cramer inferred, following Cowdry, that the secretion poured normally into the follicle, but in the hyperactive condition it passes directly into the circulation without any preliminary sojourn in the follicle. The freshly-elaborated secretion thus rapidly absorbed, they thought, might be a precocious secretion and perhaps toxic because it was precocious, which, on entering the blood stream, produced the symptomatology associated with exophthalmic goitre.

If these hypotheses were justified and the Golgi apparatus in the human thyroid gland altered its position in this fashion in health and disease, the Golgi apparatus would afford the strongest sort of morphological evidence for an exact cytology of the thyroid gland in its various morbid states and the diagnostic application of these facts would be far-reaching.

My observations on the thyroid gland of man show that the views of Cowdry, Ludford and Cramer are untenable. They are, however, becoming generally accepted in current text-books, e. g., Joll's 'Diseases of the thyroid gland,' and are

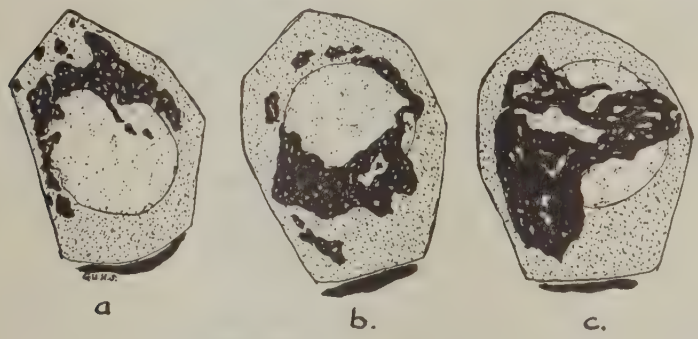

Fig. 3 Variations in the position of the Golgi apparatus of an anabolic cell when examined in serial sections of the same cell. a. The Golgi apparatus in the apical or luminal situation. b. The 'reversed' Golgi apparatus between the nucleus and the base of the cell. c. The Golgi apparatus lying across the nucleus. 5/4 x 1000.

even becoming part of current teaching. It is therefore incumbent upon us to consider the question of 'reversal' at greater length than would otherwise be necessary.

Figure 3 b is a typical example of a 'reversed' Golgi apparatus such as occurs frequently in anabolic cells, where the apparatus spreads widely. Figures 3 a and 3 c are figures of the same cell as seen in the sections immediately in front of and behind that represented in 3 b. It is obvious that the Golgi apparatus which is reversed in figure 3 b is in a 'normal' or luminal position in figure 3 a and across the nucleus in 3 c. It is therefore both reversed and not reversed in one and the

same cell. In other words it is perinuclear, but the greater amount of it is related to the apical half of the nucleus. Observations such as these depicted in figure 3 are characteristic of what was found universally in the thyroid gland whenever the trouble was taken to identify and draw a reversed Golgi apparatus from serial sections. We can, therefore, state categorically that reversal is not a fact, but that it is an appearance depending upon the perinuclear relation-

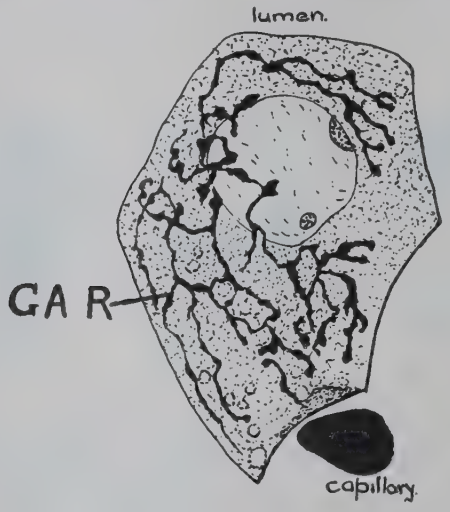

Fig. 4 Study of the Golgi apparatus after Ludford and Cramer. G.A.R., reversed Golgi apparatus. Capillary—capillary at the base of the cell.

ship of the apparatus and the particular section under consideration.

It is impossible to determine from Cowdry's ('22) pictures whether the thyroid cells he depicts are anabolic, katabolic or resting; but they appear morphologically to be anabolic cells. I will not attempt, then, to discuss his diagrams, because they correspond with those of Ludford and Cramer.

Figure 4 is a reproduction of the illustration used by Ludford and Cramer to prove the existence of the reversed Golgi apparatus in the human thyroid. It is the only accu-

rately measured illustration of reversal known. From the figure it can be seen that the cell is polygonal in outline with a regular cell membrane. The cytoplasm is heavily impregnated with a granular substance (if the stippling used by these authors in their pictures is to be taken as such) and the Golgi apparatus is very large, forming almost a complete perinuclear cap and ramifying widely in the cytoplasm. More of the apparatus appears to be situated nearer the basal than the luminal end of the cell. Such a cell is definitely anabolic, and since apparent reversal occurs in my experience in anabolic cells and it is impossible, without reconstruction, to say what is a 'reversed,' lateral or apical Golgi apparatus in such a cell, it is clear that the evidence submitted by Ludford and Cramer is inconclusive.

Reverting to the illustrations and reconstructions, figure 1, the Golgi apparatus is more intimately perinuclear in the anabolic and resting cells, whereas in the katabolic cell it has retracted slightly from the nucleus toward the lumen. Hence when anabolic (fig. 3) or resting cells are traced from beginning to end we can easily find sections in which the Golgi apparatus appears to be situated either at the apical or at the basal pole of the nucleus in one and the same cell (fig. 3 a and c), and so find evidence in a happy section (fig. 3 b) to support the migratory phenomenon of the Golgi apparatus described by Cowdry.

In the katabolic cell, on the other hand, owing to the apical retraction of the Golgi apparatus, the possibilty of including a basal fragment of the Golgi apparatus is remote. Consequently 'reversed' positions have never been described or figured in such cells. In my classification a Golgi apparatus displayed in the 'reversed' position in a katabolic cell would be incontrovertible evidence that 'reversal' of the Golgi apparatus does exist in the human thyroid gland because that is the cell which is losing its secretion. Such, curiously enough, is not the case, and only such cells as in this classification would be recognized as anabolic or resting are depicted as having a 'reversed' Golgi apparatus. If the classification

is correct, then these are precisely those cells which are not in the process of losing their secretion, and even if they had a 'reversed' apparatus, it would not be a guide to the direction in which the secretion pours.

If, then, the polarity of a cell can be determined by the position of the reticular apparatus, and reversed polarity does not exist in the human thyroid gland, the luminal position of the Golgi network and the nature of its fluctuations show that the apparatus plays a part in the formation of colloid, but does not participate in the later absorption of secretion or indicate the direction in which absorption takes place. Its position in the cell cannot, therefore, be used to show that the colloid is poured directly into the blood stream, and the symptomatology of exophthalmic goitre cannot be explained on the basis that a precocious thyroid secretion enters the circulation. Even in hyperactive thyroids there is no evidence available that reversal occurs. From the morphology of the Golgi apparatus we can only conclude that the symptoms of exophthalmic goitre are due to an excessive rather than to a precocious and perverted secretion.

CONCLUSIONS

1. In Golgi preparations, according to Da Fano's method, three distinct cell types can be recognized in the thyroid parenchyma, viz.: anabolic, katabolic and resting. The morphological criteria for this physiological classification are cell outline, cell volume, nuclear reactions, distribution of secretory granules, mitochondria and the Golgi apparatus.

2. a. The anabolic cell has a polygonal shape in section, a cell volume of 10,000 cu. μ, a centrally-placed spherical nucleus with faintly-staining nuclear membrane, numerous faintly-staining cytoplasmic granules and a large Golgi apparatus in the form of a loose network almost completely surrounding the nucleus.

b. The katabolic cell has a columnar shape in section, a cell volume of 11,000 cu. μ, a round nucleus situated nearer the apical than the basal parts of the cell, and a densely-

impregnated nuclear membrane. The cytoplasmic granules can be differentiated into three groups, one variety about the size of streptococci, giving a mahogany brown reaction with silver and situated toward the luminal pole of the cell; the second variety consisting of dark brown almost black granules about one and a half times larger than the first among which they are scattered; and, finally, a finer faintly impregnated dust-like granule located basally. The Golgi apparatus has diminished in size, is more concentrated and has retracted a little distance away from the nucleus.

c. The resting cell, the typical thyroid cell of textbooks, is cuboidal in shape, has a cell volume of 3000 cu. μ and centrally-placed nucleus. The cytoplasm is filled with a granular substance faintly-impregnated with silver. The Golgi apparatus is relatively massive and forms a nuclear cap on the luminal aspect of the nucleus.

3. The changes which occur in the Golgi apparatus of the thyroid cell during secretory activity are analogous and seemingly homologous with those changes of the Golgi apparatus which occur in exocrine glands.

4. True 'reversal' of the Golgi apparatus does not exist in normal or hyperactive (pathological or physiological) human thyroid glands. Apparent 'reversal' occurs only in single sections. Serial examination demonstrates that a 'reversed' Golgi apparatus is only a part of the complete Golgi apparatus.

5. Since 'reversal' does not exist in the thyroid cell, there is no morphological evidence that there is reversed secretory polarity in the thyroid gland.

6. The symptoms of exophthalmic goitre cannot be explained as due to a precocious secretion entering the blood stream, for even in exophthalmic goitre 'reversal' of the Golgi apparatus does not occur.

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THE OS PRIAPI: A STUDY IN BONE DEVELOPMENT

ELBERT B. RUTH

Department of Anatomy, The University of Rochester School of Medicine and Dentistry, Rochester, New York

THREE PLATES (SIXTEEN FIGURES)

Skeletal elements found unassociated with either the axial or the appendicular skeleton, whether normal or pathological in origin, offer interesting studies in bone development, which may add new data to the problem of histogenesis of osseous tissue. Instances of this type of normal bone are found in the os cordis of the ox, the os diaphragmatis of the camel, and the os priapi of several mammalian groups.

Little is known concerning the significance of the os priapi as a part of the sexual apparatus. Its appearance in certain groups of mammals, and its complete absence in other groups has been frequently observed. Its presence is extremely variable in certain orders—as a matter of fact, it may be present in some and completely absent in other members of the same order, or even the same species. As an example of this, the os priapi of the domestic cat may be cited. It varies from complete absence in some cats (or a cartilaginous nodule in some) to a well-formed bone in others (Jackson, '02). Such wide variation in the members of a single species in an order in which a well-formed bone is the rule, suggests that this structure is rudimentary in this species, and may be in the process of disappearance from the species. A genetic study of this character in the cat also might prove illuminating.

A number of theories have been offered in regard to the function of this bone—theories based upon its gross morphology and its relation to the soft tissues of the penis. However,

in view of the wide variety of forms and relations of the bone, it is obvious that no one explanation so far offered will be found applicable in all cases. Whether it fulfills only the protective and supportive function usually ascribed to bone, or whether its presence also implies some function more intimately related to the female genital apparatus is not experimentally known. It has been suggested that in those cases in which the bone exhibits an elaborate structure that protrudes beyond the limits of the soft tissues in varied hook and prong shapes, it may assist in initiating the opening of the vaginal orifice; especially, if the female shows a periodic opening and closing of the vagina during the oestrous cycle. The difficulty that arises here is due to our lack of information in regard to the oestrous cycle and the associated morphological and physiological phenomena in the females concerned. We do know the phenomena accompanying the oestrous cycle of the female guinea pig, in which there is a periodic closing of the vaginal orifice, but in this form, like the rat, the os priapi is not at all remarkable, being more or less rod-like, and wholly enclosed within the soft parts of the penis. On the other hand, the male spermophile (Citellus tridecemlineatus) exhibits a spectacular type of os priapi, which projects as sharp hooks from either side of the glans, protruding through the soft parts into the praeputial cavity. In this hibernating form the vulva of the female is small and the vagina is found to be closed throughout most of the year, with the exception of a 3 or 4 month breeding season in the spring (Johnson, Foster, Coco, '33). It seems logical to assume, however, that in this case the opening of the vagina is initiated by the same agency that controls the like phenomenon in the guinea pig. The spermophile os priapi, as well as less elaborate types of this bone may be for stimulation of the vagina or the cervix uteri, but here again we find that more knowledge is required concerning the physiology of reproduction in the forms concerned.

A certain amount of disagreement is found in relation to the position of the bone in the penis of the various mammals.

Some authors hold that it is confined wholly to the glans, and, therefore, that it may be named accurately the os glandis. Whether this is true in all cases is open to question—a question that must remain unanswered until the anatomy of the penis of each species has been carefully described. Certainly, however, the bone is confined to a position in the distal half of the penis, and has been described as occurring only in those mammals in which a glans is developed. According to Gerhardt ('05), a rudimentary glans bears only a rudimentary os penis, while the well-developed glans bears a more elaborate bone, in those forms in which the bone appears. Although this may be the rule, there are, no doubt, exceptions, and the final number of exceptions will be subject to a clear definition of exactly what constitutes a rudimentary glans penis and what a rudimentary os priapi.

The os priapi has been described in the Marsupalia, Chiroptera, Insectivora, Rodentia, Carnivora, Cetacea, and in the Primates. It varies in length from a few millimeters in some of the smaller mammals (or where it appears to be more or less rudimentary) to 55 cm. in the walrus and 2 m. in the whale. The bone has never been observed in the Ungulates; and although no discussion is found in the literature concerning its appearance or non-apperance in the Monotremes, it is exceedingly doubtful whether it occurs, because of the primitive type of genitalia found in this group.

The presence of a penis bone in man has been discussed by several authors, who have described it as being more or less rudimentary, and appearing intermittently in the white race and the black race. According to these observers, the bone present in the human penis is better developed among the negroes. Such discussions are found principally in the earlier literature on the subject, one author going so far as to describe the presence of bone in the human penis as a constant morphological character, but adding that true bone may not always be demonstrable, in which case at least a cartilaginous nodule would be present. It is elementary knowledge at present that bone is not found in the normal, healthy human penis.

The observations of earlier writers who described bone present in the human penis, were no doubt made in cases where heteroplastic bone was present. Such instances are by no means rare, and have been observed and described many times in recent literature. These cases may be traced to some pathological condition, as it is well known that bone sometimes appears in syphilitic gummata; following trauma with extravasation of blood; or following an inflammatory condition such as cavernitis. Jacoby ('24) describes the bone found in man as a 'fascia bone' occurring in the fibrous tissue of the tunica albuginea. It arises as a series of calcareous deposits between the fiber bundles of the fibrous tunic. These fuse into a homogeneous mass, wherein the connective tissue cells become bone cells, and in which haversian canals and lamellae are present. Osteoblasts or osteoclasts are not present, neither is there cartilage formation. It is therefore apparent that bone in the human penis cannot be considered an atavistic structure homologous with the os priapi of the Prosimii and Simiidae, and the mammals below the Primates. While the os priapi of lower mammals represents a periosteal-osteoblastic formation of a definite form and size for each species, showing typical developmental processes in the young; in the human penis it must be considered a fibro-metaplastic progressive development of a wholly abnormal structure.

The literature yields numerous descriptions of the penis bones of the various mammals, and in several papers their taxonomic significance has been pointed out. As yet little use has been made of these bones in differentiating between species. Relatively little attention has been given to their developmental history; factors governing their growth, repair, and regeneration; or to their physiological significance.

There is considerable disagreement as to the events occurring in the developmental history of the bone. This suggests the possibility of a slight difference in the sequence of developmental phenomena in different mammalian groups, or the probability of error in the interpretation of these phenomena. One author describes an enchondral origin, and observes that

the mesodermal elements differentiate into cartilage, which is replaced by bone. Another considers it to be essentially intramembranous in origin, but adds that there is undoubtedly some cartilage in relation to its development, and suggests that there is still need for further investigation.

This discussion is concerned with the development of the os priapi of the white rat. The rat was chosen, obviously, because it is a convenient laboratory mammal to work with, both because of its size, and the ease with which different developmental stages can be obtained. Similar investigations are in progress on the guinea pig, the spermophile (Citellus tridecemileatus), and the white-footed mouse (Peromyscus manniculatus).

Grossly, the os priapi of the adult white rat is a small styloid bone averaging about 3.5 mm. in length. In transverse section it is seen to vary from oval to round, and tapers from its wider, slightly flattened proximal end, or base, to its bluntly pointed distal end, or apex. From the base, which is 1.8 mm. in diameter, it narrows rapidly in the proximal fourth, and then more gradually toward the apex, which is 0.5 mm. in diameter. It is located in the distal half of the penis, above the urethra (fig. 16). At the proximal end a cartilaginous nodule is present and continuous with the bone, while at the distal end a mass of dense fibrous tissue consisting of closely woven bundles of collagenous fibers bears a similar relationship to it. The cartilaginous nodule at the proximal end becomes progressively smaller, until it can scarcely be demonstrated in old adult animals.

The os priapi of the rat develops relatively late in ontogeny, as does the penile part of the urethra and other morphological characters of the definitive penis. Up to late pre-natal life the mesenchyme of the phallus shows very little differentiation. Shortly before birth the formation of the penile portion of the urethra is completed, and the anlage of the os priapi is indicated. Several stages of development from the first appearance of the anlage of the bone to its development into the definitive structure have been chosen for description.

Twenty-day fetus. The penile part of the urethra at this stage is formed only in the proximal two-thirds of the phallus and is closed externally and inferiorly only by a thin wall of flattened epithelium, two cells in thickness, which is continuous externally with the outer layer of the skin and internally with the epithelium of the urethra. This layer of cells closes the urethra from the proximal end to the distal extent of this incompletely formed urethral canal. Beyond this a vacuolization of the cells indicates that the opening up of the urethra is imminent. The anlage of the os priapi is indicated by the condensation of the mesenchymal cells which appear as a mass of deeply stained nuclei just above the position that will be occupied by the urethra, in the distal half of the penis. At this time no differentiation is yet apparent in the regions to be occupied by fibrous tissue, bone, or cartilage in the definitive structure. Spaces containing nucleated erythrocytes are present on either side of the anlage of the os priapi, the primitive cavernous spaces (fig. 1).

One-day-old rat. The urethra is patent throughout its extent. Above it there is still little apparent differentiation of the densely grouped mesenchymal cells which occupy the position of the os priapi and its associated structures. It is at this stage, however, that the appearance of osteoid substance is first demonstrable. Toward the center of the mesenchymal cells occupying the position of the bone, a slight change in arrangement of the cells can be observed. The peripheral cells and their nuclei become flattened, and lose the appearance of being placed at random. They are seen to be arranged circularly about the central core of mesenchymal cells. These latter appear to show signs of enlargement, and at the same time an amorphous osteoid substance that stains a light red with eosin appears more or less irregularly among the cells—apparently a product of a secretory activity of these cells (figs. 2 and 4). There is no differentiation of fibrous tissue or cartilage in the penis at this period of development. The mesenchymal cells of the core of the bone anlage have assumed an osteogenic capacity, which, by their continued

secretory activity separates them farther and farther apart. As the intercellular substance increases in amount it stains a darker red with eosin. The nuclei of the cells also become more deeply stained, and the cytoplasm is much reduced. The cells about the periphery of the osteoid axis assume more angular shapes just internal to the circularly arranged spindle-shaped cells surrounding the axis. The cells enclosed within the osteoid mass are not arranged regularly, or in lacunae, but may be placed three or four cells together in a space, loosely surrounded by it, or perhaps one cell may be isolated here and there. The osteoid substance shows a fenestrated structure, in which the cells appear to be placed at random. At both the proximal and the distal end of the area of ossification the deeply stained nuclei are densely grouped, and show no appreciable differentiation.

Growth in length and circumference is continued, in the manner described, for the first 3 days, during which time the bone attains a length of approximately 0.5 mm., with slight variations. In longitudinal section the osteogenic cells, at each end of the area of ossification, at times indicate a slight tendency to line up in a manner somewhat recalling the column formation of the cartilage cells during enchondral ossification. The cells about the periphery of the bone are differentiated into two types—an outer layer of flattened spindle-shaped cells which will form the definitive periosteum, and an inner layer of angularly shaped more or less stellate osteogenic cells, the osteoblasts. The original osteogenic cells that elaborated the osteoid substance, and are now entrapped within it, are the bone cells (fig. 3).

Four-day rat. By the fourth day the structure has the appearance of true bone. It is limited by periosteum about its periphery. The bone cells are now relatively widely separated by intercellular osseous matrix—each cell in its own lacuna. The mesenchymal cells at the proximal end have differentiated into chondroblasts, and the process of specialization of these cells soon allows a cartilaginous area to be distinguished, and a base of hyaline cartilage is in this way

formed. This differentiation of these cells is observed in the staining reaction (the hyaline matrix taking the hematoxylin) as well as in the histological picture in which the cells show the typical arrangement in lacunae. The central cells have a peculiar appearance, being large, clear, and granular. The cartilage is continuous distally with the bone, and indeed, the breakdown of the large vesicular cells appears to be intimately related to the formation of the medullary cavity in the proximal end of the bone, and to the linear extension of the bone from this time on. This is the first appearance of cavity formation, and occurs at the same time that some of the more centrally located bone cells seem to exert an osteolytic activity, thus widening their lacunae until the walls between adjacent lacunae are thinned and broken down, allowing several spaces to coalesce. This at first forms a cancellous structure and later a marrow cavity by the continued osteolytic activity. At intervals the bone is invaded by periosteal buds, and near its center a primitive and limited marrow cavity is present (fig. 5). Primitive vascular elements can be observed, and osteoclastic activity is readily demonstrated. At this time the cells at the distal end of the bone are specializing as fibroblasts and the formation of the dense fibrous tip can be seen (figs. 6 and 7). Circumferential growth is carried on by the formation of external lamellae through subperiosteal osteoblastic activity, while growth in length is accomplished from the proximal end by replacement of the cartilage there present, by bone.

Ten-day-old rat. No outstanding changes are observed up to this time. Now, however, capillaries and erythrocytes appear in the medullary cavity, which is being gradually extended. At the proximal end the bone is cancellous, and this tissue gives way to the medullary cavity in the middle third. The medullary cavity, surrounded by compact bone, narrows distally into a small canal in which vascular elements are present. Several diverticula lead out from the medullary cavity and parallel with it in the compact bone. The bone continues to gain greater circumference through appositional growth by sub-periosteal osteoblastic activity.

Fifteen-day-old rat. The cancellous nature of the proximal end of the bone and the cartilage with which it is continuous are strikingly apparent at this stage (figs. 8, 9, and 10), and the fibrous cap at the distal end of the bone is well differentiated. The dense fibrous bundles blend with a fibro-cartilaginous tip which is continuous with the bone. The central cells of the fibro-cartilage are vesicular and tend to break down as they meet the bone, where the spaces formed by their disintegration are continuous with the marrow cavity which now extends from one end of the bone to the other.

Thirty-five-day-old rat. At this age the bone has attained practically its definitive condition as far as progressive developmental phenomena are concerned, except for the attainment of its full length and circumference. It is not a series of haversian systems, but it is rather one large system in which the medullary cavity represents the haversian canal containing fatty and vascular elements. This in turn is surrounded by lamellae and lacunae in which the bone cells lie. The medullary cavity is for the most part centrally located, but its caliber varies greatly, from 0.09 to 0.5 mm. As has been stated several diverticula from it extend out into the compact bone, and in this way bring the vascular supply nearer to the outlying bone cells (figs. 11, 12, 13, and 14). The proximal end remains cancellous in structure as long as the bone is growing in length, bony trabeculae extending in all directions from the peripheral compact layer. When the full length is reached and the cartilaginous proximal end is no longer being invaded by the osteogenic processes, the bony trabeculae are no longer present, and even the cartilage gradually disappears, as its presence is no longer necessary. During the period of growth, however, vascular elements—especially erythrocytes, are much more numerous at the proximal end where greater metabolic activity is apparent.

Six-months-old rat. The adult os priapi presents a well-developed pad of dense fibrous connective tissue at its distal end, and continuous with it. The shaft of the bone is surrounded by a fibrous periosteum—the layer of osteoblasts

being no longer present. What appears to be a layer of endosteum with well-developed cells lines the medullary cavity in which capillaries are supported by a connective reticulum. Myeloid elements are scarce through most of the shaft, but at the proximal end become more abundant. Throughout the shaft the lamellar structure of the bone is easily apparent, especially as the periphery is approached. In the proximal end vascular elements and fat cells are numerous. Sagittal section through the proximal end, or base, of the bone shows cartilage to be absent, its function being no longer apparent after the bone has reached its definitive length (fig. 15).

DISCUSSION

As been pointed out, there is a disagreement among the few authors who have made observations on the development of this bone, as to its origin. Three views have been advanced—1) that it is intra-membranous in origin; 2) that it has an enchondral origin; and, 3) that it arises from membrane, but has some relation to cartilage; the last view suggesting the need for further investigation.

Intramembranous ossification implies the presence of a membrane of some sort, or at least of demonstrable fibers or fibrils, as the precursor of bone formation. Shipley ('28) says, concerning membrane bone,

> the development occurs in connective tissue membranes . . . . certain cells develop into osteoblasts and form an osteoid ground substance which becomes calcified. At the same time a change takes place in the fibers of the connective tissue. Straight radiating bundles are formed which are continuous with the ordinary fiber bundles at their extremities. Osteoblasts migrate along these fiber bundles depositing ground substance as they go, which becomes calcified to form true bone. The osteogenic fibers, so called, are identical in composition with ordinary white fibers. In this manner embryonic bone is formed of irregular spicules radiating from a central mass. The spicules become joined to each other by branches and a network of bone is formed. The formation of osteogenic fiber bundles precedes the advancing growth of the spicules of bone. The interstices between the spicules

are filled with connective tissue in which myeloid tissue develops, and with blood vessels and osteoblasts. The interstices continually become narrowed by accretions of bone newly laid down on the old spicules. In the meantime a periosteum has formed on either side of the cancellous tissue, and the outer and inner tables of the fully developed bone are formed by this membrane.

Ham ('32) is somewhat more conservative in his terminology, using 'osteogenic cells' to designate these first lining cells of the fibrous tissue, instead of 'osteoblasts,' since these cells appear to be able to form either bone or cartilage, under proper conditions. He also observes that cartilage formation in the course of intramembranous ossification is rare, being confined normally to the temporo-mandibular articulation. Bardeen ('10) observes that a rich vascular supply arises concomitantly with the formation of the bony spicules, forming a network of spicules interwoven with a network of blood vessels.

The question of whether the os priapi is of enchondral origin need not be considered, since the bone is well established before the cartilage is differentiated. In regard to its relationship to intramembranous bone, however, several points must be considered. There is an absence of connective tissue fibers when the bone is first laid down; it forms as an homogeneous osteoid mass that extends more or less evenly in all directions, perhaps extending proximally and distally at a slightly higher rate; there is no spicule formation; formation of cells that are typically osteoblasts in their position and morphology is relatively late; vascularization is slow, not occurring until several days after birth; formation of periosteum and subperiosteal bone is tardy; cartilage is normally present after the fourth postnatal day, and takes a seemingly active part in the formation of the definitive structure.

Maximow ('31), describing the formation of membrane bone, says, "Very rarely there are no fibers in the connective tissue where the bone develops, and the substance of the bars is homogeneous, but even here a fibrillar structure soon appears." He does not name the membrane bone in which

this absence of fibers has been observed in its early development, nor say whether this may occur now and then in the development of any of the membrane bones.

Intramembranous ossification illustrates a simpler type of bone formation than does enchondral ossification. In the os priapi an even simpler type is found, if the degree of differentiation of the skeletogenous mesenchyme is accepted as a criterion for simplicity. Osteogenic cells become active in laying down osteoid substance before any marked differentiation of surrounding tissues can be observed—an *endoblastemal ossification.* Here, in an interesting manner, is seen an illustration of the potentialities of the mesenchymal cells, wherein three different types of tissue (fibrous, cartilaginous, and osseous) are developed concomitantly, and in close relation to each other, from the same area of condensation of undifferentiated cells.

SUMMARY

1. The skeletogenous area is indicated by a condensation of the mesenchymal cells.

2. Without further differentiation the osteoid substance is laid down by these cells, and becomes calcified.

3. At the distal end of the bone anlage dense fibrous tissue is differentiated; and hyaline cartilage is formed, at the same time, at the proximal end.

4. Growth in length is accomplished by substitution at the cartilaginous base; and circumferential growth by addition of lamellae through subperiosteal osteoblastic activity.

5. This bone is of endoblastemal origin, and illustrates a remarkable type of histogenetic acceleration.

6. A marrow cavity, in which myeloid elements are present, is formed by resorption of the osteoid tissue, which results first in the formation of a cancellous structure, and finally a medullary cavity.

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PLATE 1

EXPLANATION OF FIGURES

1 Transverse section through the penis of a 20-day-old fetus. The anlage of the os priapi is seen as a darkly stained condensed area of mesenchymal cells just above the anlage of the urethra. × 100.

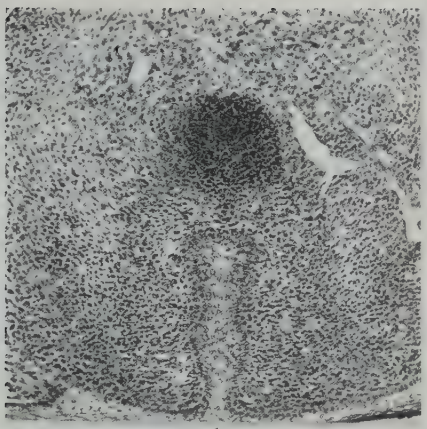
2 Transverse section through the penis of a 1-day-old animal. The osteoid tissue of the os priapi is seen above the urethra. × 100.

3 Longitudinal section through the penis bone of a 2-day-old animal. × 100.

4 Transverse section through the os priapi of a 1-day-old animal. Same section as figure 2. × 430

5 Transverse section through the os priapi of a 4-day-old animal, showing the invasion by a periosteal bud and vascular elements. × 100.

6 Transverse section of the same bone as figure 5, showing an osteoclast, osteoblasts, and vascular elements.



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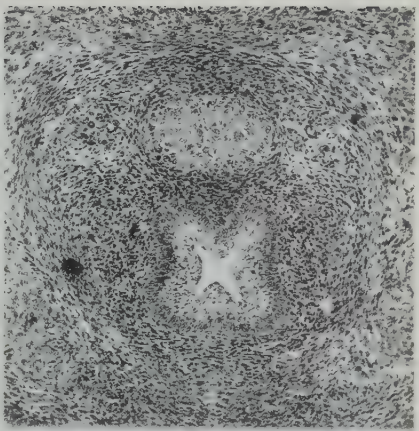

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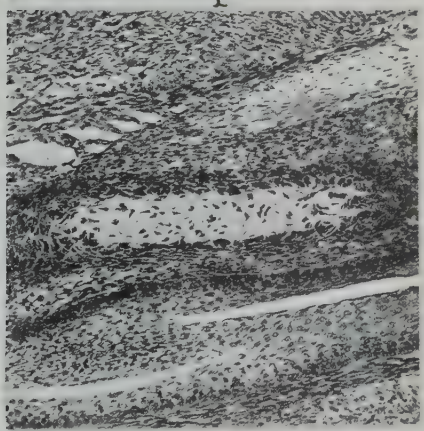

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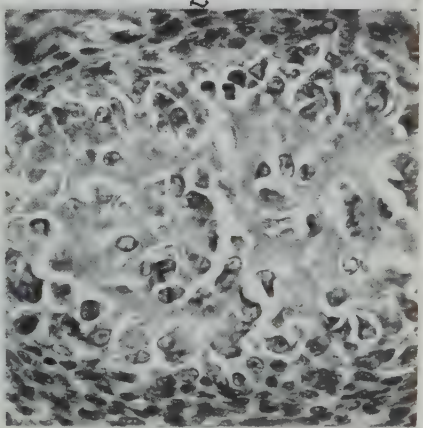

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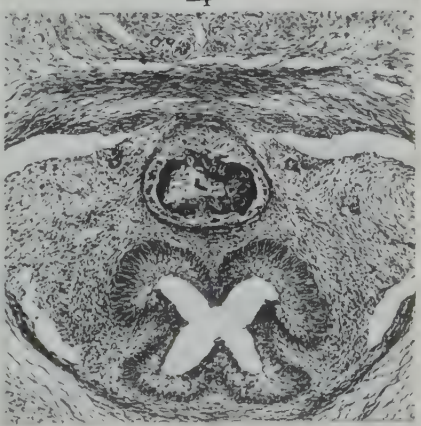

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PLATE 2

EXPLANATION OF FIGURES

7 Transverse section through the distal end of the penis of a 4-day-old animal, showing the differentiation of the fibroblasts and the formation of the fibrous tip. × 100.

8 Transverse section through the proximal third of the penis of a 15-day-old animal, showing cancellous structure at this end. × 100.

9 Transverse section through the hyaline cartilage continuous with the proximal end of the bone of figure 8. × 100.

10 Longitudinal section through the proximal end of the os priapi of a young adult, in which the bone has achieved the definitive size and form. Note relation of bone and cartilage, and the absence of cancellous structure. × 100.

11 and 12 Transverse sections through the os priapi of a 35-day-old animal, showing differences in caliber of the medullary cavity. The osteoblastic layer of the periosteum is plainly seen where the fibrous layer has shrunken away. × 100.

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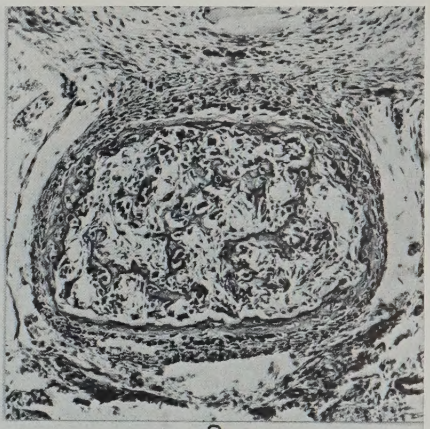

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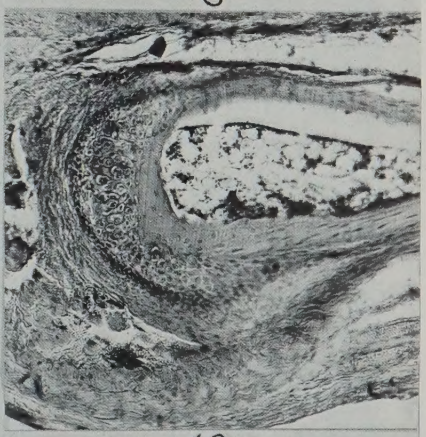

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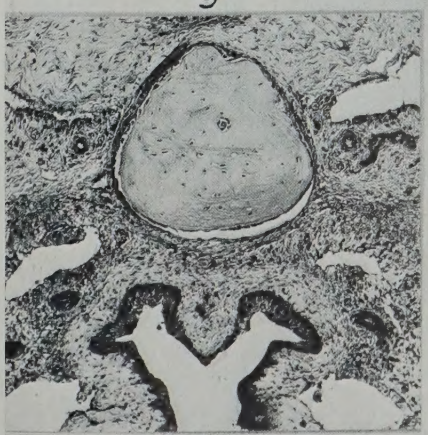

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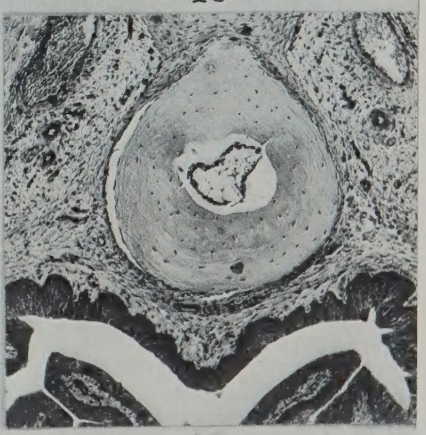

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PLATE 3

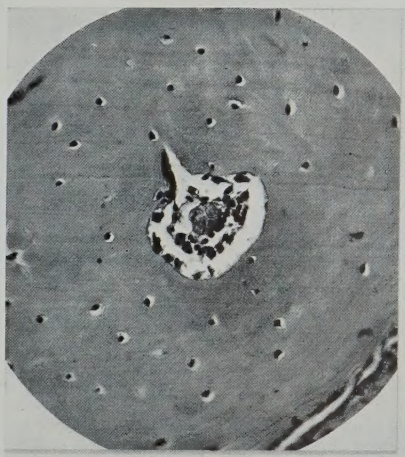
EXPLANATION OF FIGURES

13 Transverse section of the same bone as figures 11 and 12, under high magnification. Myeloid elements, a capillary, and endosteum are plainly seen. × 250.

14 Transverse section through the os priapi of a 6-month-old animal. × 250.

15 Longitudinal section through the proximal end of the os priapi of a 6-month-old animal. The cartilage at the proximal end has disappeared. × 250.

16 Several types of penis bones: 1) Mus norvegicus albinus; 2) Mustela vison; 3) Procyon lotor; 4 and 5) Canis familiaris 6) Euarctos americanus.



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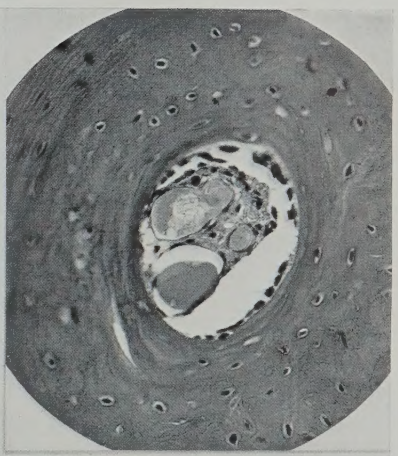

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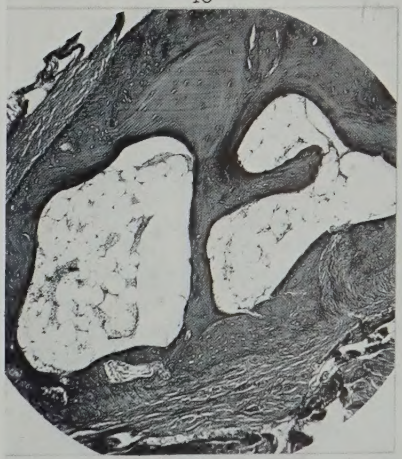

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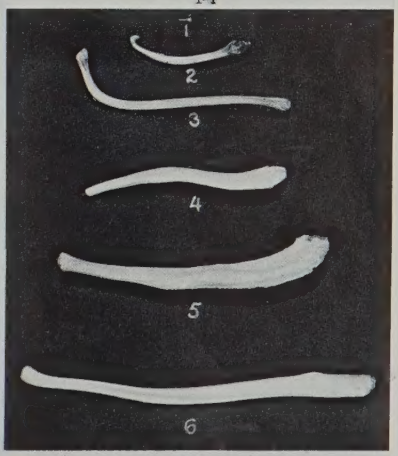

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